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Cultured intestinal epithelial cells (I-407) and standard tissue culture techniques were used to study the following: a) the necessity of changing culture medium the day following subculture; b) the normal growth curve of an early and older passage of cells; c) the alterations in the growth curve of the cells after a 1 hour pulse or exposure with 17 β-estradiol, vitamin A, estradiol-vitamin A combination, alcohol control, and untreated control; and d) differences in cellular response to therapeutic and ethanol-diluted sources of 17 β-estradiol.

Determinations of the number of viable and non-viable cells/ml in cell suspensions were made involving cellular fixation with a 2.5% solution of glutaraldehyde. Cell proliferation was determined by hemocytometer counts.

Concentrations of 17 β-estradiol and vitamin A were chosen for this in vitro study based on normal physiological levels. A review of the literature pertaining to these test substances and procedures for determining cell growth and proliferation is included.

The critical point in cell proliferation when the culture medium was not changed was found to be at 48 hours. Growth curves for cells cultivated under normal conditions were plotted for periods of 72 hours. Control and alcohol control cell treatments exhibited a significant difference only at the 48 hour point after the 1 hour exposure. No significant difference in cell proliferation took place due to the

Progynon estradiol; presence or absence of Progynon also had no effect on cell proliferation. Ethanol-diluted estradiol elicited a highly significant cellular response, and the presence or absence of the estradiol was significant. From these results, it was concluded that ethanol-diluted estradiol was preferable for *in vitro* work. Vitamin A administered alone resulted in a decreased cell number. When statistically analyzed with the Progynon, there was no significance in its interaction with time; however, this interaction of vitamin A with time was significant when analyzed with the ethanol-diluted estradiol. The analysis of variance indicated an interaction between Progynon and vitamin A when administered in combination and no interaction between time and this combination. The interaction of the ethanol-diluted estradiol and vitamin A showed no significance in the analysis of variance; but the interaction of time, estradiol, and vitamin A was significant.

A chromosomal examination of typical cultured cells revealed an average number of 77.5 chromosomes per cell. This was in agreement with the aneuploid modal number of 76 reported for this cell line. A typical chromosome spread and karyotype are included.

THE EFFECT OF 17 β-ESTRADIOL AND VITAMIN A ON THE PROLIFERATION OF THE CELL LINE I-407

by

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CHAPTER I

INTRODUCTION

Proliferation and differentiation of the cells of an organism are essential processes for proper growth and function. Cell proliferation of human cells begins with the first cleavage of the zygote. Some tissues or cells cease their proliferation or division once they have differentiated into their ultimate morphologies and functional duties, while other tissues are noted for their continual cell division and renewal. Cell proliferation and differentiation are greatly influenced by substances produced by organs or tissues within the human body as well as substances which must be provided by external sources. One such chemical substance synthesized by particular organs or tissues is the steroid sex hormone estrogen. Vitamin A is termed an essential dietary substance since it cannot be synthesized by any organ or tissue of the human body, although it may be regarded as a hormone in that the liver maintains the amount in circulation (Wolf and DeLuca, 1970). Estrogen and vitamin A are complex molecules carried via the bloodstream to all body tissues. The manner in which many cells differentiate is largely determined by the presence or absence of these as well as other substances. The primary interest of this research was the role played by both estrogen and vitamin A in controlling cell proliferation.

Estrogen is a steroid produced by the ovaries under the control of pituitary gonadotropins. Normal physiological levels of estrogen, bound with high affinity to an estrogen-binding protein in the blood, have the

general effect of promoting tissue growth, primarily in accessory sex tissues. Estrogen stimulated cell proliferation has been noted in the oviduct epithelium (Socher and O'Malley, 1973), human endometrium and myometrium (Chen, Lindner, and Lancet, 1973), and rat uterine epithelium (Katzenellenbogen and Gorski, 1972).

Vitamin A, a fat-soluble vitamin, plays an important role in the maintenance of proper physiological conditions, including maintaining rhodopsin for the rods and cones in the eyes and regulating bone growth and epithelial tissue differentiation (Wolf and DeLuca, 1970). The vitamin A or retinol circulates in human plasma bound to a specific retinol-binding protein which has prealbumin mobility on electrophoresis (Goodman, 1969). A deficiency of vitamin A alters the epithelial tissue by decreasing the number of mucus-secreting cells whereas the reverse occurs in the presence of excess amounts of the vitamin (DeLuca, et al., 1972). Vitamin A administration to tracheal and gastrointestinal cells exerts a control over the ability of cells to undergo tumorigenic or malignant transformation (Saffiotti, 1969). This is in addition to controlling their ability to switch their differentiation from mucoprotein synthesis to keratin synthesis. Observations by Lasnitzki (1961) also showed vitamin A stimulation of cell proliferation in vaginal epithelium.

Since both estrogen and vitamin A are bound to specific protein molecules and carried throughout the body in the blood, all human tissues are subjected to both substances, though the response of various tissues to these substances varies. The tissue studied in this investigation was the epithelium of the intestine. Studies of intestinal epithelium

in the rat crypts of Lieberkühn (Wright, Morley, and Appleton, 1972) revealed that the varied cell positions exhibited varied durations of mitosis with the entire cell cycle time at the bottom of the crypt being greater than 20 hours. Mazia (1974) stated that the length of the cell cycle of a given kind of cell, at optimum temperature and nutrition, is always the same. Since an established tissue culture cell line of intestinal epithelial cells was used for this study, the purposes of the investigation were:

1. To determine the need for changing the culture medium the day following subculture, presumably to remove toxic by-products and/or to replenish growth factors.

2. To establish the normal growth curve of the cultured cells, first, of cells in an early passage, and secondly, of cells which had been cultivated over a longer period of time.

3. To determine if, and in what manner, a 1 hour exposure to 17 β-estradiol, vitamin A, estradiol-vitamin A combination, alcohol control, and untreated control may alter the growth curve of the cells.

4. To observe differences in cellular response to a therapeutically used source of 17 β-estradiol and an ethanol dilution of 17 β-estradiol.

5. To establish a technique of chromosome analysis for comparison of karyotypes at various cell passages.

CHAPTER II

REVIEW OF LITERATURE

The review of literature for this investigation includes a more in-depth study of the nature and mechanisms of action of the two major test substances, estrogen and vitamin A. In addition, various techniques for measurement of cell growth were reviewed. Two Medline computer-based searches of the literature were carried out through the facilities of the Health Sciences Library at the University of North Carolina at Chapel Hill.

Estrogen

The predominant natural estrogens of the human are 17 β-estradiol, estrone, and estriol, with 17 β-estradiol and estrone being interconvertible and estriol being the predominant urinary end-product of estrogen metabolism (Turner and Bagnara, 1971). The primary sites of estrogen action are the secondary sex tissues of the female. Proliferation of the epithelium of the fallopian tubes is induced by estrogen. Increased growth and mitotic activity in the myometrium, endometrium, and mammary gland, and increased secretions of the uterus and vagina are also caused by estrogen (Heftmann, 1970; O'Malley, 1971).

Obvious estrogen target tissues like those of the uterus and vagina possess the ability to concentrate and retain the hormone, without chemical transformation (Bresciani, et al., 1973). Tissues such as the liver and muscle display no binding receptors on plasma membranes but contain a concentration of estrogen in proportion to the concentration of the

estrogen bound to plasma proteins in the blood (Blyth, et al., 1973; Gorski, et al., 1967). Geynet and associates (1972) also pointed out the belief that estradiol, the predominant free form of estrogen, enters all non-target cells but flows back out of them freely, after the initial diffusion. Bresciani and associates (1973) explained that the estrogen-binding proteins on cells bind in-coming hormone in the cytoplasm. The estradiol-receptor complex undergoes a change and moves through the cell to the nucleus where it interacts with the chromatin. Only a few minutes are required for the complete disappearance of the hormone from the cytoplasm and concentration in the nucleus. Bresciani, et al. (1973) noted a strong indication that estrogen acts at the nuclear level. This is in line with one of the earliest cellular responses to the hormone being an increased transcription rate.

The process of the translocation of the protein-bound estrogen from the cytoplasm to the nucleus has been shown to be temperature-dependent. It is during this translocation that the protein undergoes an alteration, or "receptor transformation" (Jensen and DeSombre, 1973). This transformation is noted by an increase in sedimentation rate and ability to bind to isolated uterine nuclei. A tissue specific deficiency in the RNA synthesizing capacity of such nuclei is also alleviated (Jensen and DeSombre, 1973). O'Malley and Means (1974) also suggested that one of the biochemical functions of estradiol may be the induction of receptor protein conversion to an active form capable of entering the nucleus, binding to acceptor molecules, and inducing RNA synthesis. The estrogen may even result in changing the chromatin template which allows an increased number of binding sites for RNA polymerase (O'Malley and Means, 1974).

An estrogen receptor is required for the initiation of cell proliferation by estrogen, and estrogens may act by their ability to induce and maintain cell proliferation in tissues (Bresciani, et al., 1973). It has been noted (Socher and O'Malley, 1973) that estrogen is necessary to maintain a high frequency of cell division. This is particularly noted in the surface epithelium of the immature oviduct which is stimulated by estrogen to divide. The data from their experiments also indicated that estrogen may stimulate a single population of cells to divide. Chen, Lindner, and Lancet (1973) also reported that the addition of estradiol to the culture medium of human endometrial and myometrial cells caused an increase in the rate of cell division and thymidine incorporation into DNA. In vivo administration of estradiol to mature rats was found (Chen, et al., 1973) to induce only epithelial mitoses in the uterus. Proliferative stimuli may act to instigate entry of cells in the prereplicative G1 phase into the DNA synthetic S phase, G2 phase, and then mitosis or possibly to stimulate cells from G2 into mitosis. Studies with fluorodeoxyuridine or hydroxyurea along with the hormone determined that the stimuli causing proliferation acted prior to the completion of DNA synthesis, thus in G1 or S (O'Malley and Means, 1974). They also stated that G1 cells are apparently stimulated by estrogen into the S phase. Socher and O'Malley (1973) also reported that estrogen stimulates a resting G1 or G_o population of cells in the surface epithelium of the oviduct to initiate DNA synthesis and divisions.

Riggs (1970) stated that estrogen may act directly on the tissue to change transport. It was reported that steroids, such as estradiol, are able to alter the permeability of artificial lipid spherules to anions,

cations, glucose, or glycine. In vivo, 17 β-estradiol stimulates the transport of both sugars and amino acids into rat uteri. Just as it may act on the permeability of lipid spherules, estradiol is also known to provoke lysosome-membrane instability (Szego, et al., 1971). Further work by Szego (1972) noted that cortisol blocks estrogen-induced lysosomal membrane destabilization. Szego (1972) also came to the conclusion that lysosomal enzymes and acidic glycolipoprotein matrix from hormone-destabilized organelles may derepress the genetic apparatus. Intranuclear and nucleolar penetration of fragmented lysosomal components has been noted (Sansone-Bazzane, et al., 1972) via electron microscopy within 15 minutes after estrogen administration to ovariectomized rats. Premature intracellular destruction may be due to lysosomal enzyme release following estrogen administration.

Vitamin A

An important member of the group of fat-soluble vitamins is vitamin A. Although it cannot be synthesized by the human body, vitamin A performs four basic physiological functions which are as follows (Wolf and DeLuca, 1970):

1. Maintenance of proper vision.
2. Maintenance of spermatogenesis in the male and prevention of resorption of the fetus in the female.
3. Maintenance of bone growth, in particular, the activity of the osteoblasts and, therefore, prevention of deficiency-caused nerve disorders due to pressure of overgrown bone on nerve cells.
4. Maintenance of the mucus-secreting cells of epithelia and prevention of keratinization.

It is well known that vitamin A and its derivatives aid in the maintenance of eye tissues and pigments and therefore of proper vision. Studies on rats and chicks by Thompson (1969) confirmed that vitamin A was needed

for fetal development. Retinoic acid administration to rats did not hinder good growth or conception, but the fetuses were resorbed. Johnson, Kennedy, and Chiba (1969) reported a failure of response to dosages of estrogen in female vitamin A-deficient rats.

Vitamin A is a complex molecule of formula $C_{20}H_{29}OH$ with five conjugated double bonds and a terminal primary alcohol group. Due to the alcoholic structure, this compound can form esters and be oxidized to an aldehyde. The basic carbon skeleton arrangement of the conjugated double bonds cannot be altered or there is a loss of biopotency (Ames, 1969). Ganguly (1969) explained that the absorption of vitamin A means the absorption of retinol and its esters, retinal and retinoic acid. Roels (1967) noted that the dietary retinyl ester is hydrolyzed in the lumen of the intestine before passing across the mucosal cell wall. The vitamin A absorbed by the intestine is transported via the intestinal lymphatics. Experiments confirmed that retinyl esters predominate in the intestinal lymph after rats were fed retinol of β-carotene (Huang and Goodman, 1965). The retinyl ester is hydrolyzed into retinyl palmitate which is stored in the liver before passing to body tissues via the blood. Studies with human plasma have shown that a retinol-binding protein is the substance responsible for vitamin A circulation in the blood. Muto and Goodman (1972) noted that the binding of the retinol to the retinol-binding protein (RBP) is physiologically important since the interaction solubilizes the water-insoluble retinol molecule and protects the retinol molecule against chemical degradation. Each RBP has a binding site for only one molecule of retinol, and the RBP has no fatty acid or fatty acyl chains. The retinol transport in plasma thus appears to

involve both a lipid-protein (retinol-RBP) interaction and a protein-protein (RBP-prealbumin) interaction (Goodman, 1969). This protein-protein interaction seems to partly stabilize and protect the protein-bound retinol molecules, and it also prevents the glomerular filtration of the RBP molecule and its loss in the urine.

Intracellular levels of vitamin A are determined by its transport across the cell membrane. This therefore effects the amount of vitamin A available for biological function. A deficiency of vitamin A effects RNA synthesis at the ribosomal RNA and transfer RNA levels (DeLuca, et al., 1971). In the absence of vitamin A, ribosome activity in the liver increases. An increase in protein synthesizing activity is traced to low molecular weight t-RNAs and synthetases (C & EN, 1972). Zachman (1967) noted that possible sites of vitamin A action leading to an increase in RNA synthesis may be that: 1) the vitamin may act as a cofactor in an essential metabolic sequence; 2) the site of vitamin action may somehow be related to energy production; 3) the vitamin may stabilize a membrane structure which is essential for synthetic reactions; 4) the vitamin may act as a depressor and thereby stimulate a specific RNA synthesis.

Vitamin A has the unique potential to induce and control epithelial differentiation. Its specific morphogenetic effect on epithelium is noted as one reason for the interest developmental biologists have in vitamin A (Barnett and Szabao, 1973). DeLuca and Wolf (1970) noted that in vitamin A deficiency, mucus-secreting tissues are drastically altered due to a basic change in the pattern of basal cell differentiation from mucus to keratinizing. DeLuca, et al. (1972) also stated that squamous metaplasia occurs due to a vitamin A deficiency, a process similar to

that induced by chemical carcinogens. The transformed epithelia are characterized by the loss of tonofilaments and inhibition of keratinization, disorientation of basal and lower stratum spinosum cells with an increase in the size of intercellular lacunae, general lack of cell density, and formation of microvilli at the outer surface of the epidermis (Barnett and Szabao, 1973). While the deficiency of vitamin A causes a cessation of growth in the epithelium of the epidermis and keratinization of most mucus-secreting tissues, a vitamin A deficiency in intestinal mucosa results only in degeneration of the mucin-secreting goblet cells. It was also pointed out (DeLuca, et al., 1972) that chemical carcinogens also only produce adenocarcinomas of the intestinal mucosa without any squamous changes. In exact opposition to the deficiency of vitamin A, an excess amount causes keratinizing tissues to become mucus-secreting and display typical goblet cell morphology. By using polyribosomes from intestinal mucosa cells, it was shown that the rate of protein synthesis was not effected by a deficiency of the vitamin. This was measured by the uptake of labeled amino acids (DeLuca and Wolf, 1970). It was also noted (Zachman, 1967) that the administration of retinol increases the mitotic index of both deficient and normal epithelial tissues.

DeLuca, et al., (1971) believed that the primary action of vitamin A in the differentiation of mucous cells is at the level of specific glycopeptide synthesis. It has also been shown that vitamin A is required for the synthesis of the type of mucopolysaccharides secreted by the colon. By a process of elimination of other steps in the mucopolysaccharide synthesis, it was concluded that the vitamin A functions either in the polymerization of the UDP derivatives or the activation of transfer of

sulfate to the polymer (Wolf and Varandani, 1960). Willmer (1961) noted that vitamin A is involved with the manner in which the mucopolysaccharides are deposited either inside or outside the cells. High doses were stated to favour the production or storage of mucosubstances and the uptake of inorganic sulfate by epithelial cells. These high doses can also cause the complete resorption of the ground substance of cartilage by the cartilage cells. DeLuca and Wolf (1970) also reported that from a variety of experiments with several different tissues it seems clear that vitamin A is needed for a very basic and general step in the biosynthesis of glycoproteins. In addition, it seemed likely that vitamin A functioned as a sugar carrier lipid in the cellular membrane for biosynthesis of glycoproteins (DeLuca and Wolf, 1970). Baume, et al., (1970) noted that vitamin A also may modify the activity of certain enzymes, such as acid phosphatase, and possibly within lysosomes. Willmer (1970) suggested that one of the actions of vitamin A is to increase the permeability of phospholipid membranes. This may be the mode of action on the lysosomes, in agreement with Szego (1972) when she stated that vitamin A has also been implicated in the destabilization of cellular membranes. Action of vitamin A on the permeability of the mitochondrial membrane has also been noted (Baume, et al., 1970).

Lasnitzki (1961) reported the effect of the presence of vitamin A on vaginal epithelial tissue as increasing cell number and size, provoking the formation of keratohyalin, and suppressing keratin synthesis. The presence of estrone, however, is reported to slightly stimulate changes in squamous cells such that more basal and transitional cells are formed (increased cell division) and more keratin is laid down. However, if an

effective level of vitamin A is present, it can antagonize the estrone action and prevent the keratinization of the vaginal epithelium. A relationship between *in vivo* action of vitamin A and estrogen is therefore established. Another such relationship was drawn from a study of women taking oral contraceptives (Gal, et al., 1971). It was found that there was a significant increase in circulating vitamin A in women taking contraceptive pills, thus suggesting that exogenous steroids have a significant influence on vitamin A metabolism. It was also noted that administration of vitamin A and exogenous steroids are able to induce congenital malformation in animals, and in relation to this is the observation that higher post-partum levels of plasma vitamin A were found in women who have borne children with central nervous system defects than women with normal children (Gal, et al., 1971).

Vitamin A exerts its effects in individual cells and the cellular synthesis of polypeptides and other cell products such as mucopolysaccharides. From its effects on individual cells, vitamin A then exerts its effects on entire tissues. Several relationships have also been noted to exist between the actions of vitamin A and estrogen on epithelial cells. Both substances stimulate cell division and differentiation of cells, but this differentiation is in opposition. Vitamin A causes cells to form mucus secreting goblet cells whereas the lack of vitamin A results in keratinization. Estrogen causes keratinization of cells when it is present, an action which is suppressed by vitamin A administration. Both substances also destabilize membranes such as those of the lysosomes. Thus, a comparison of the ways in which estrogen, vitamin A, and the

combination of the two substances may alter the normal growth of cells not considered to be estrogen target cells is of interest.

Techniques of Cell Growth Measurement

In order to compare the growth of untreated cells with estrogen and/or vitamin A treated cells, a method of determining cell proliferation must be chosen. Growth in tissue culture has been roughly defined as an increase in the number and mass of the cells (Parker, 1961). Under some conditions, an increase in the number of cells may take place without an increase in mass, and it is also possible to note a mass increase without cell number increase. In the past, growth measurements were made in various ways including: 1) periodic measurements of the surface area of the cultures; 2) estimates of the ratio of resting cells to dividing cells in an area of the culture at a particular time; 3) cell weights after separation from plasma coagulum by chemical means; and 4) measurements of chemical changes taking place in the culture system (Parker, 1961). The measurements of surface area required periodic measurements and construction of diagrams with the increase in area expressed as a function of time. This method, however, fails to distinguish between increases in area resulting from cell multiplication and increases due to cell migration. Variations in the density of the cell population, the general thickness of the colony, or the cell size are also not accounted for by this method. In many instances, it is not possible to make serial cell number measurements for following the growth of cultures, in which case it may be helpful to determine the mitotic coefficient. The mitotic coefficient is the ratio of dividing cells to resting cells and is done by determining the number of mitoses per 100 cells. A method such as

this is dependent on being able to examine the culture with phase contrast optics at high magnification or by alternatively fixing and staining the culture (Merchant, Kahn, and Murphy, 1960). Time lapse photography may also be used to estimate the percentage of dividing cells per unit of time, expressed as the mitotic index. Reliance on the mitotic index as a judge of the mitotic rate may lead to incorrect conclusions as well since some treatments may alter the length of phases of the cell cycle, thus resulting in a higher or lower than normal concentration of dividing cells at the time under study.

A change in total cell mass as determined by changes in dry weight may be a useful device for following in vitro development of some cell populations. An increase in dry weight indicates an increase in cell mass. By combining the measurement of packed cell volume and cell number with the measurement of dry weight, a calculation of net synthesis and changes in degree of hydration at the cell and population levels is made possible (Merchant, et al., 1960). The chief limitations are the time consumption and necessity for rather large samples in order to obtain accurate determinations. More biochemical assays of total nucleic acid content and proteins also are indicators of cell growth in vitro, however, variations in mean content of protein, RNA, and DNA are such that no one measurement can serve as a reliable measure of population development.

Merchant, et al., (1960) pointed out, if cells can be grown as monodisperse suspensions or can be dispersed adequately and maintained as a stable suspension, cell counting techniques are preferred. The hemocytometer count is noted to be the only feasible means of routine cell count in most laboratories, despite the amount of variation. It is, as

Parker (1961) pointed out, always important to know the percentage of viable and non-viable cells in a population. A number of vital staining procedures have been developed as a means of distinguishing between living and dead cells on the basis of selective permeability to dyes. The amount of error in using the hemocytometer for determining the percentage of viable and non-viable cells in a population is recognized. It is acknowledged that obtaining a total cell count from an electronic cell counter is more accurate and faster, but no distinction can be made as to viable and non-viable cells. For this reason and the lack of availability of such a cell counter, determinations of cell proliferation were made following the dye-exclusion methods and hemocytometer count.

CHAPTER III

MATERIALS AND METHODS

An established line of human intestinal epithelial cells, designated I-407, was obtained from the American Type Culture Collection Cell Repository (Rockville, Maryland). Standard tissue culture techniques (Merchant, Kahn, and Murphy, 1960) were employed throughout the investigation. The medium used for growth of the cell monolayers was Eagle's Minimum Essential Medium (MEM) with Earle's balanced salt solution (Grand Island Biological Company; American Type Culture Collection, CCL6) containing necessary salts, stable amino acids, vitamins, glucose, and phenol red indicator. Fetal bovine serum (FBS) (GIBCO) was added in varying percentages to provide a source of amino acids, glucose, and a growth stimulating factor. Temin (1972) noted that the cell multiplication is controlled by the availability of these growth stimulating factors in the serum. Since serum appears to play an important role in the adherence of the cells to the culture surface, the cells were initially placed in medium containing 15% FBS. Also added to the basic medium and FBS solution were non-essential amino acids (NEAA), a less stable amino acid L-glutamine, and the antibacterial agents streptomycin and penicillin (GIBCO; media formulations for MEM and NEAA found in GIBCO catalog). These substances were added in the following amounts, with the 5 percent level of FBS used for cultivation under experimental conditions being indicated:

MEM with Earle's BSS	95 ml
FBS	5 ml
NEAA	1 ml
L-glutamine	1 ml of 100X stock, 2mM final concentration
Pen-strep	1 ml of 10,000 units and 10,000 µg/ml stock

Although the tissue cultures were started on medium with 15% FBS, they were gradually decreased to 10% FBS and then to the 5% FBS level at which the experiments were carried out. This was done to deplete the cells of possible trace amounts of estrogen and vitamin A in the serum. When the cells, which were cultivated in 25 cm^2, 30 ml Falcon polystyrene flasks, had grown to cover most of the bottom of the flask, a confluent monolayer was judged to exist. The cultures were then harvested, or subcultured, and divided into portions which were placed in new flasks. Subculturing was done by washing the monolayers twice with phosphate buffered saline (PBS - Ca^{+} and Mg^{++} free) and the cells detached with a 0.25% trypsin solution in PBS. Cultures were incubated at 37°C with the trypsin solution approximately 5 minutes, and then each flask was jarred to complete the cell detachment. Subculturing and dilution enabled the cells to continue maximum possible growth because of removal of the restriction of limited space. Hemocytometer (Spencer Bright Line Hemocytometer) counts of the cells were made to obtain desired cell concentrations at the onset of each experiment.

Choice of Estradiol Concentration and Source

Svendson and Sorensen (1964) reported the normal adult female estrogen level in the blood as being 0.0001 to 0.001 µg/ml. During pregnancy, estradiol was estimated to be at a level of 0.015 to 0.030 µg/ml. Birth control pills of the 2 mg size add a maximum of 0.1 mg of

estrogenic component to 5,000 ml of blood, thus representing a final concentration of 0.02 µg/ml. This 0.02 µg/ml concentration was noted to be an intermediate estradiol level during pregnancy and was therefore chosen for this study. To establish a system most like that in the human body, a therapeutic source of 17 β-estradiol was chosen (Schering Corporation's Progynon aqueous estradiol suspension). This source provided a sterile suspension of crystalline estradiol in isotonic saline with 0.025 mg polysorbate 80 and 0.5% phenol as preservative. The stock solution was in a concentration of 1,000 µg/ml, and sterile PBS and culture medium were used for further dilution to the final concentration.

Anhydrous 17 β-estradiol (Sigma Chemical Company) was dissolved in 95% ethanol to make a stock solution of 1,000 µg/ml which was diluted with PBS and medium and membrane filtered to obtain the sterile final 0.02 µg/ml concentration. This form of 17 β-estradiol was also tested to note any possible variations in action in an *in vitro* system from those noted to be caused by the therapeutic estradiol source. Differences in cellular response to the estradiol could then be accounted for by the presence of the detergent-like substance, polysorbate 80, and the powerful preservative, phenol, in the therapeutically used estradiol suspension.

Choice of Vitamin A Concentration and Source

Physiological concentrations of vitamin A were desired for use in this study. In determinations of the vitamin A concentration in human tissues from five areas of the United States, it was reported (Raica, Scott, Lowry, and Sauberlick, 1972) that the tissue vitamin A level was dependent on the concentration of vitamin A in the liver. The average liver vitamin A concentration for 372 samples was reported from the above

mentioned experiments to be 146 ± 151 µg/g. The tissue vitamin A concentration, excluding liver tissue, was found to be approximately 1 µg/g, or 1 µg/ml, of wet tissue. Previous experiments indicated that a vitamin A level of 5 µg/ml was optimum, although in some conditions this may be considered toxic. This was the concentration chosen for study. A level such as this, which would be greater than the normal tissue vitamin A level of 1 µg/ml, would also be desired in order to elicit a cellular response to the vitamin A. Anhydrous vitamin A in the all trans-retinol configuration (Sigma Chemical Company) was dissolved in 95% ethanol to make the 100 mg/ml stock solution which was further diluted to 5 µg/ml in the culture medium. Sterilization of the solution was accomplished by membrane filtration (Swinny Millipore, 0.22µ). All procedures and flasks involving the vitamin A solution were protected from light to prevent excessive break-down.

Preliminary Experiments

Preliminary experiments to determine the probable action of the substances on the cells were performed. Monolayers of cells were subcultured and pooled to provide an evenly distributed cell suspension. A count was made of the number of cells/ml that were distributed in 1 ml quantities to sterile 16 x 125 mm Leighton tubes (Bellco Glass Company) containing coverslips (11 x 35 mm size). Within 24 hours, the medium was changed to remove any cell fragments and toxic materials that may have been present from the subculturing. Forty-eight hours after subculture, the estradiol and vitamin A solutions were added. One coverslip was removed as a 0 hour control. For the time periods of 24, 48, 72, and 96 hours, one tube was treated with unaltered control medium, one with estradiol, one with

vitamin A, and one with equal amounts of the estradiol and vitamin A solutions. Each day at a designated time, the coverslips from the appointed tubes were removed and fixed in Carnoy's fixative (1:3 glacial acetic acid and methanol). After removal of the 48 hour coverslips, the media with the appropriate test substances was changed on the 72 and 96 hour tubes of cells. The coverslips were fixed, stained with Giemsa stain, and mounted on slides. Fifteen microscopic fields were counted for statistical purposes. The fields were chosen at random, and counts were made of each field to determine the total number of cells.

Major Experiments

The basic methods chosen to establish the growth curves in the major portions of the investigation involved the growth and trypsin removal of cells, fixation, vital staining, and hemocytometer counts. From previous determinations of optimum cell proliferation in various size flasks, it was found that a cell concentration of 7.5×10^5 cells/ml insured cell proliferation in the Leighton tubes (Curtis, 1973). To obtain this concentration, cells grown in the 25 cm^2 Falcon flasks were subcultured and partially diluted with 5% FBS medium. Cell suspensions from all flasks were pooled, aspirated vigorously to prevent clumping, and 0.5 ml of the suspension was added to 1.0 ml trypan blue. The number of cells/ml in the suspension were counted and calculations were done to determine the amount of medium to be added to adjust to a concentration of 5×10^5 cells/ml. Each Leighton tube received 1.5 ml of the above suspension, corresponding to 7.5×10^5 cells. No coverslips were present in the sterile Leighton tubes. Three replications of the experiment were set up and carried out simultaneously to insure identical experimental conditions.

The basic procedure followed in each experiment involved the removal of the cells grown in the tubes by the usual trypsinization technique of subculturing. One ml amounts of cell-trypsin suspension were removed from each tube via pipet and placed in a small histological vial. An equal amount of a 2.5% glutaraldehyde in PBS solution was added to each suspension. This procedure was based on dye-exclusion tests for cell viability following fixation which were reported by Yip and Auersperg (1972). In accordance with their techniques, the fixed cell suspensions could be placed in an ice bath for 1 hour and then into 4°C or else directly into 4°C. Vital staining was done with 0.1 ml of 0.3% alcian blue and 4.5% ETOH dye plus 0.9 ml of the fixed suspension. Three cell counts of each sample were then made. Their results indicated that the number of cells which took up the dye, an indication of cell death or non-viability, increased slightly between 1 hour following fixation and 24 hours following fixation. There was, however, no significant increase in stain uptake from 1 day after fixation until approximately 1 week after fixation. A similar check on the staining properties of the I-407 cells was made for 1, 24, 48, and 72 hours after fixation and findings are reported in the results section.

No medium change. The purpose of the first experiment which was carried out was to determine the need for changing medium following the subculture of cells. The cells used for this portion were from passage number 276 (P-276). The pooled cells were put in nine Leighton tubes, and the medium was not changed 24 hours following the subculture as in normal procedures. The cells were removed and fixed 24, 48, and 72 hours following their inoculation into the tubes. Three counts of the fixed

cells were made the day following fixation, and the number of viable and non-viable cells/ml calculated.

Establishment of normal growth curve. The purpose of the second two-part experiment was to establish the normal growth curve of the I-407 cells with (1) cells from an early passage number and (2) cells from an older passage which had been cultivated over a longer period of time. For part 1, cells from P-276 were used in setting up the nine tubes necessary for the three replications of the 24, 48, and 72 hours counts of the cells. Cells from P-296 were likewise used for part 2. The only point of variance in standard procedures was that the 72 hour counts for part 2 were made after only 1 hour of fixation, whereas all previous and subsequent cell counts were made the day following fixation.

Test variables. The major portion of the investigation was to determine whether a 1 hour exposure to 17 β-estradiol, vitamin A, and a combination of 17 β-estradiol and vitamin A would effect the growth curve of the cells. Katzenellenbogen and Gorski (1972), using a 1 hour exposure time, demonstrated that induction took place of the synthesis of the induced protein in rat uteri in vitro within 40 minutes. Rao (1969) determined an estradiol-induced mitotic delay following only 15 minutes of exposure to estradiol, and early large bursts of RNA synthesis in responsive cells were also noted within 1 hour of the estrogen pulse (Bresciani, et al., 1973). Since most investigators assume saturation of cellular binding sites by estradiol within a few minutes, and because of the mechanics of maintaining asepsis during and after administration of the substances, the 1 hour exposure time was chosen. It should also be noted that no information on binding site saturation time for vitamin A

was found in the literature reviewed. Cells from P-281 were pooled and proper concentrations of cells inoculated into each of the 45 tubes. The following day, dilutions were made in 5% FBS medium of 17 β-estradiol (Progynon), vitamin A, and 95% ethanol. The designated tubes of cells were given 1 ml of the Progynon, vitamin A, Progynon-vitamin A combination, alcohol (ethanol) control, and untreated control solutions. These substances remained on the cell cultures for 1 hour, were aspirated off, and were replaced with control medium. The cultures were then incubated at 37°C until the designated tubes of cells were removed 24, 48, and 72 hours after the exposure to the test substances. It should be noted that these times are taken to be "following treatment" rather than "following subculture" as was the case in previous experiments to establish the growth curve. Standard procedures of cell removal, fixation, and making 3 counts per sample were followed.

Results from the cultures subjected to the Progynon 17 β-estradiol created an interest in how the cells would respond to 17 β-estradiol in ethanol solution with no additional preservatives. Three replicates using this form of estradiol were set up with cells from P-283. The combination of this estradiol and vitamin A was also tested using cells from P-285. All subsequent procedures remained unchanged. Any differences in response to the two forms of estradiol could then be attributed to the preservatives in the Progynon solution.

Chromosomes. A technique for chromosome analyses was also developed for the cells in use even though a statistically complete chromosomal analysis of the cells was not carried out. The procedure was adapted from that of Grand Island Biological Company and is as follows:

1. Grow the cells in either 25 cm^2 or 75 cm^2 Falcon flasks with 5 ml of culture medium.

2. Add 0.3 ml Colcemid Solution (10 µg/ml; GIBCO Catalog #521) to the cell culture. This yields a final concentration of 0.6 µg/ml Colcemid.

3. Incubate at 37°C for 2-3 hours.

4. To harvest the cells from the culture flasks, use either a rubber policeman or an improvised rubber policeman to scrape off the monolayer of cells into the medium.

5. Transfer the cell suspension to a centrifuge tube - may rinse the flasks and utensils with PBS to insure obtaining a maximum number of cells. Centrifuge at 1000 rpm (160 x g) for 10 minutes (International Centrifuge, Model HN).

6. Discard all but 0.25 - 0.50 ml supernatant. Wash the resuspended cells with PBS to remove any serum remnants and centrifuge at 1000 rpm (160 x g) for 6-10 minutes.

7. Discard all but 0.25 - 0.50 ml supernatant and resuspend cells in remaining supernatant.

8. Add the hypotonic solution of 0.075 M KCl (GIBCO Catalog #R15-0575) to a total volume of 4 ml.

9. Centrifuge at 750 rpm (90 x g) for 6 minutes. Do not allow cells to remain in the hypotonic solution longer than 12 minutes.

10. Discard all but 0.25 ml supernatant and resuspend cells.

11. Add 4 ml freshly prepared Carnoy's fixative (1:3 glacial acetic acid and methanol).

12. Allow to stand 15 minutes at room temperature.

13. Centrifuge at 750 rpm (90 x g) for 6 minutes.

14. Discard all but 0.25 ml supernatant; resuspend cells; repeat addition of 4 ml fixative followed by 15 minutes at room temperature. Centrifuge at 750 rpm (90 x g) for 6 minutes.

15. Discard all but 0.50 ml supernatant. Resuspend pellet.

16. To make slides, place 2-3 drops on a clean, chilled, wet slide held at a 45° angle. Water on the slide spreads the cells on the surface or else blow gently to spread.

17. Examine unstained slides under phase to determine if a more concentrated suspension is needed.

18. Stain with Giemsa stain.

Photographs were made of the cells in culture through an Olympus (Model CK) inverted phase microscope. A Wild (Model M11) microscope was used for all other microscopic work and photomicrographs. A 35mm Nikon camera was used on these microscopes with Panatomic X film, ASA 32, for black and white photos. Color slides were made with Kodak's high speed Ektachrome tungsten film, ASA 125. Color prints were then made from the slides. Luminos resin coated paper, contrast #3, was used for the printing of black and white photographs.

The Olivetti Underwood Programma 101 calculator was used for part of the calculations necessary for the analyses. The computer program TSAR, Telestorage and Retrieval system, written at Duke University, was used for all statistical computations.

CHAPTER IV

RESULTS

Preliminary Experiments

The preliminary experiments involved the enumeration of the stained cells on mounted coverslips. The total number of cells per field was determined; and from the 15 fields counted on each slide, the mean number of cells per slide was calculated. For views of cells grown on coverslips, see figures 1, 2, 3, and 4. The initial number of cells/ml inoculated into the Leighton tubes containing the coverslips varied. The average initial number of cells inoculated into the culture tubes was 376.3×10^3 cells/ml. For these experiments, the test substances remained on the cultures for the entire incubation period, and the medium with the test substances was changed 48 hours after the initial inoculation. The resultant growth curve (figure 5) showed a steady increase with the growth slackening between 48 and 96 hours after treatment. Treatment with Progynon estradiol resulted in a slower increase in cell number than the control until it almost reached the control level at 48 hours. Following the renewal of the medium with the estradiol at the 48 hour time, a decrease in cell number occurred, followed by another rise in total cell number. Cell growth after vitamin A treatment was similar to that of the estradiol. The 24 hour level was somewhat higher than the 48 hour estradiol point. The two lines then parallel each other to the 96 hour time, with vitamin A being the lower of the two. The combination treatment resulted in a 24 hour level of cells slightly higher than the control.

Figure 1. Color Photograph of Giemsa Stained Cells

Cells grown on coverslips and stained with Giemsa stain as in preliminary experiments. Note cell in telophase of mitosis. 400X microscopic magnification and 740X total magnification.

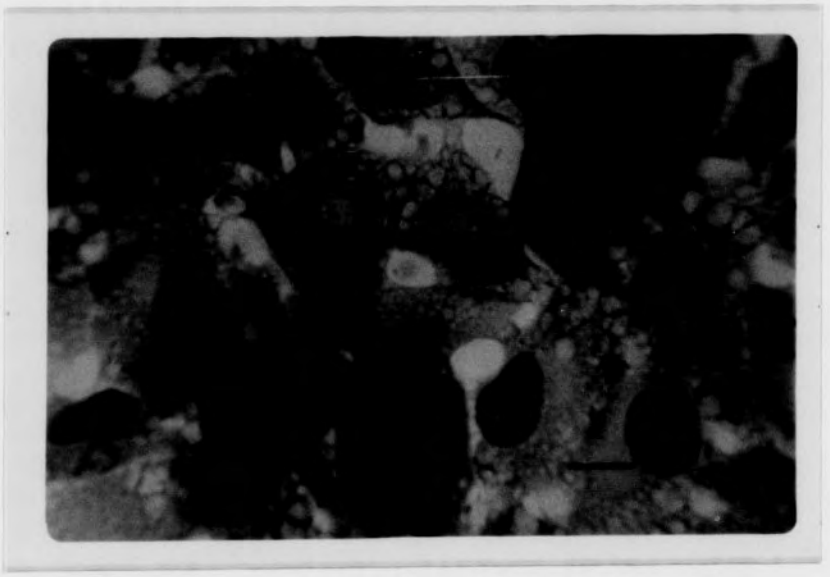


Figure 2. Black and White Photograph of Cells on Coverslips

Note epithelial morphology and mitotic cells. 400X microscopic magnification and 740X total magnification.

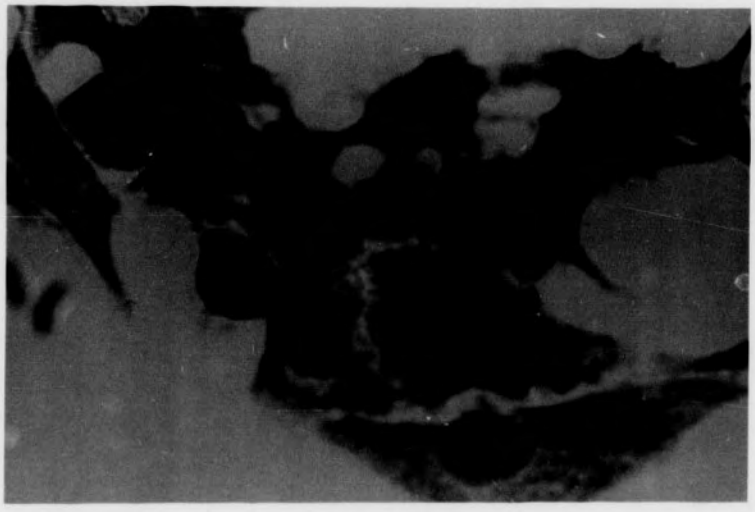


Figure 3. Color Photograph of Cells Stained with Cell Cycle Stain

Note binucleate cell, cells in prophase and telophase stages of mitosis. 400X microscopic magnification and 740X total magnification.

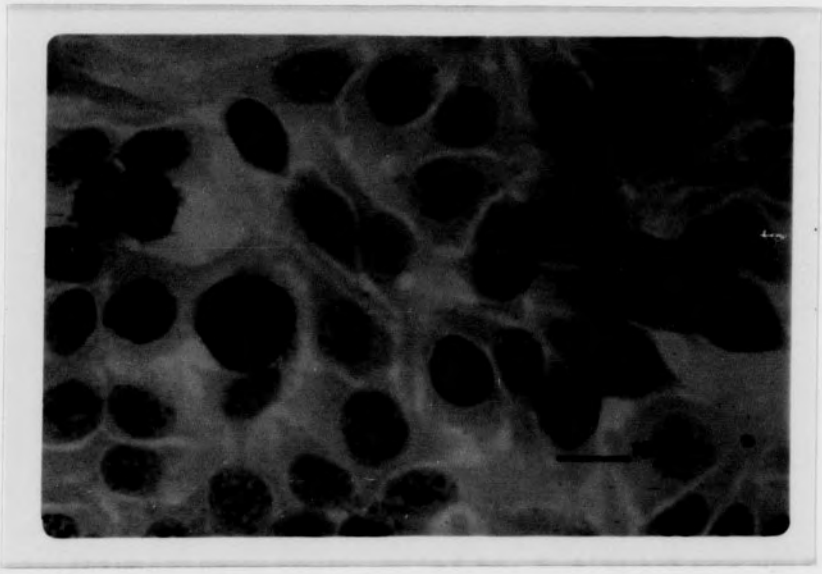


Figure 4. Telophase Cell

Cells on coverslip were stained only with a solution of acridine orange in sucrose. 400X microscopic magnification and 740X total magnification.

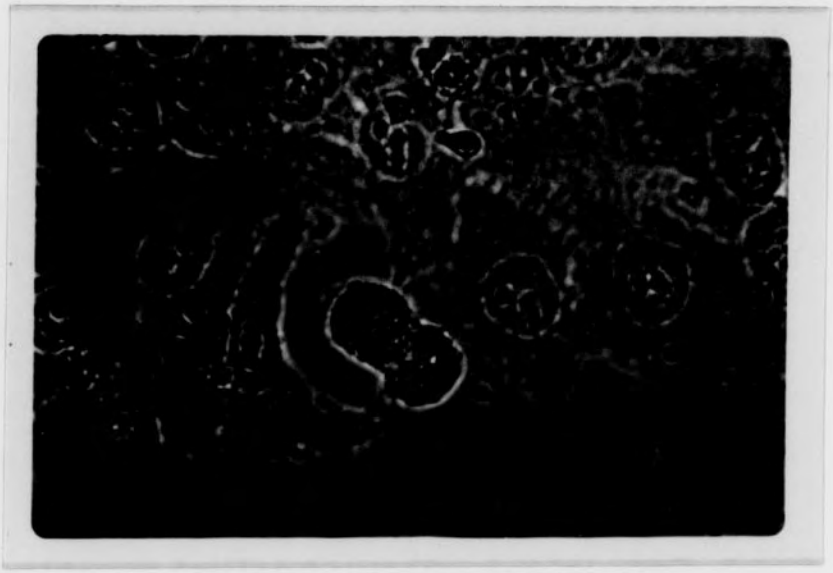
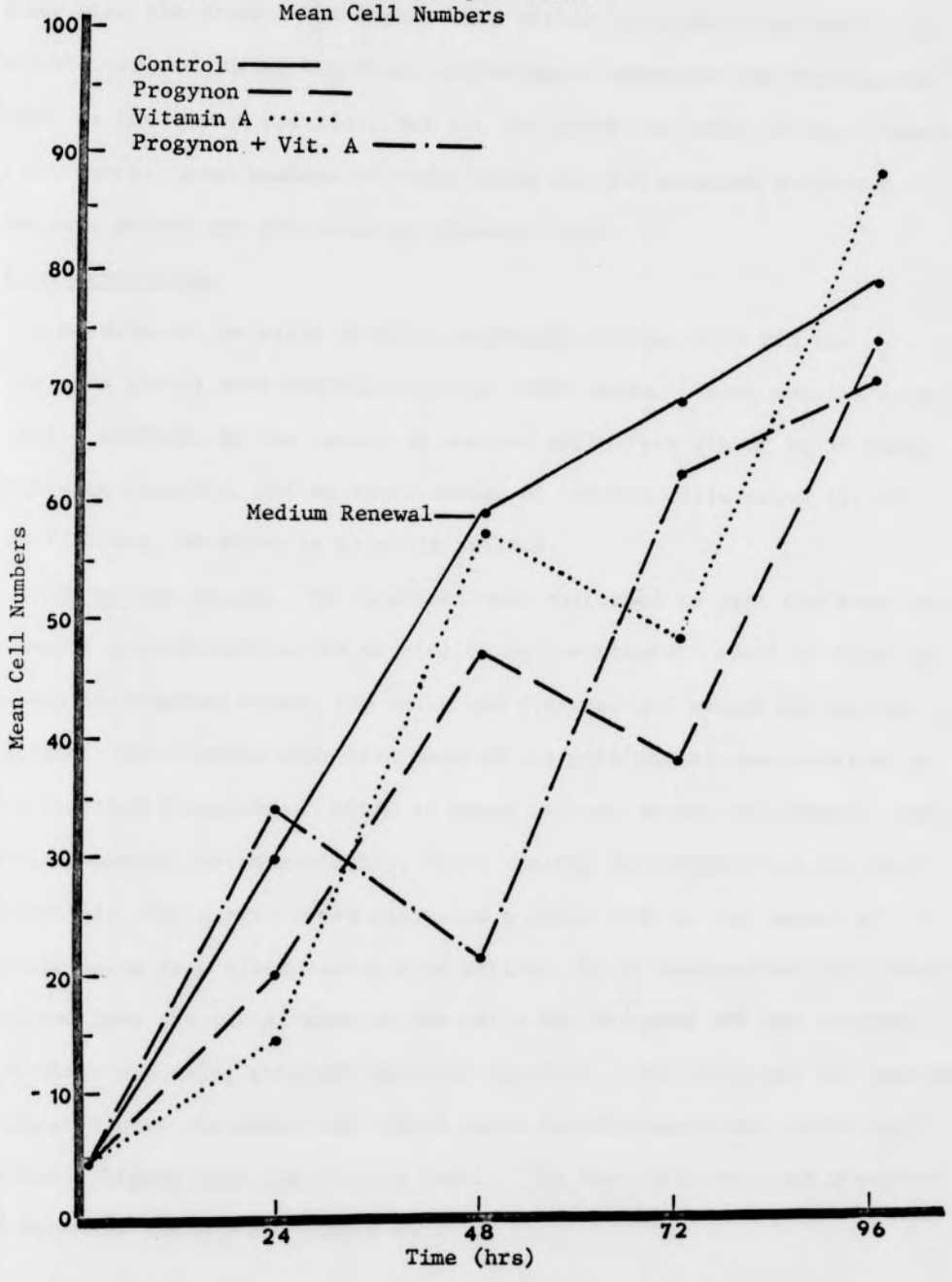


Figure 5. Preliminary Experiments
Mean Cell Numbers



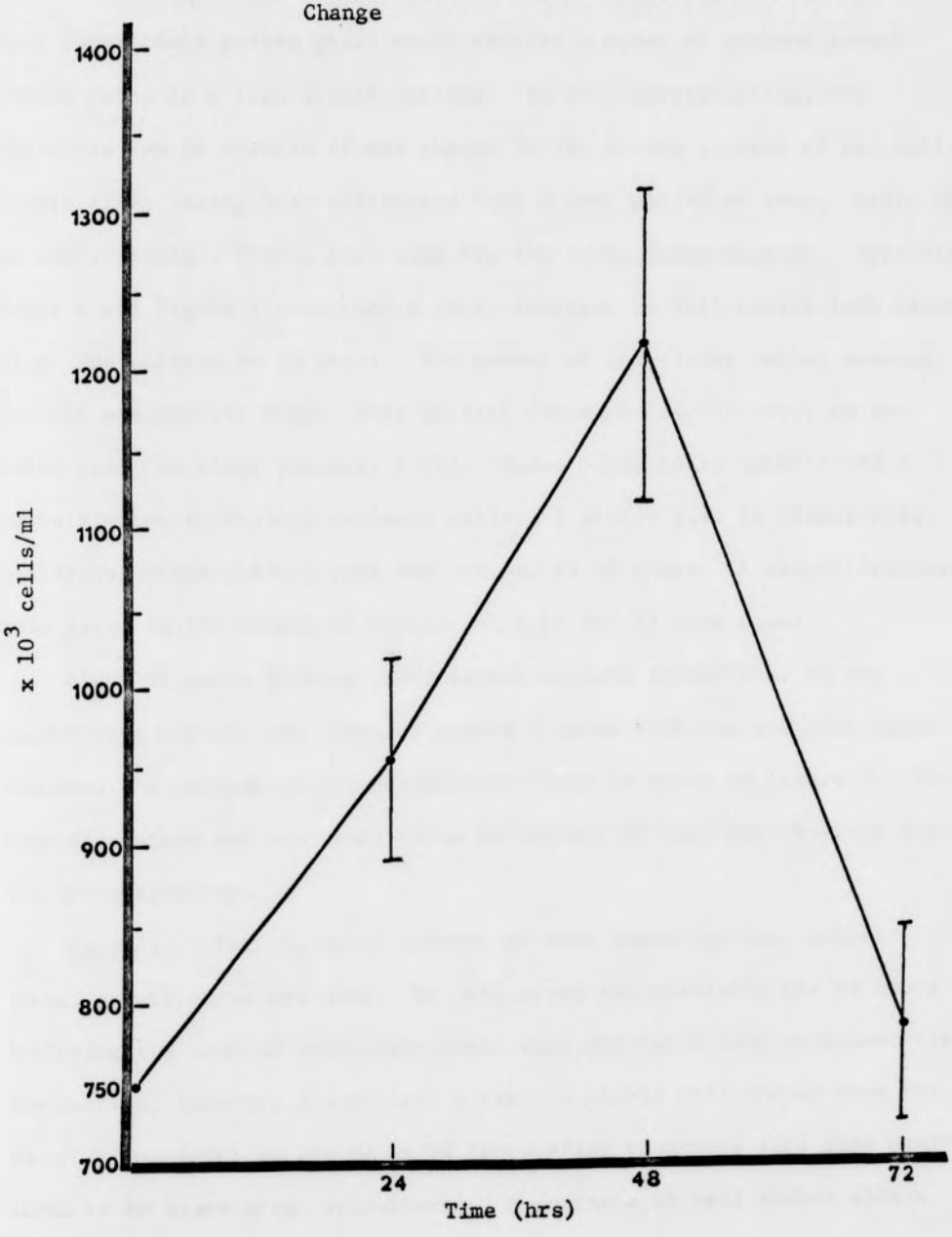
By 48 hours, a decrease occurred; and by 72 hours, the cell number was lower than the control but higher than either individual treatment. A slight increase during the final period again delegated the combination level to the lowest position, but not far below the level of the vitamin A treatment. Mean numbers of total cells and the standard deviation of the data points are presented in Appendix table 1.

Major Experiments

Results of the vital staining technique adopted from Yip and Auersperg (1972) were verified for the I-407 cells. These results were: a small increase in the number of stained cells from 1 hour to 24 hours following fixation, and an equal number of stained cells after 24, 48, and 72 hours, as shown in Appendix table 2.

No medium change. An experiment was performed to test the need for changing culture medium the day following subculture. After 24 hours of growth in Leighton tubes, the cells had attached and spread out on the surface. Cell counts were then made of the cell suspensions obtained by the standard procedures. After 48 hours without medium replacement, the cells appeared, microscopically, to be healthy and normal, and had proliferated. The growth curve indicates a sharp rise in the number of viable cells took place during this period. By 72 hours after the inoculation into the tubes, many of the cells had sloughed off the surface, and those remaining attached appeared abnormal. The counts of the samples indicated that the number of viable cells had decreased to a point only slightly higher than the initial level. The resultant data are presented in Appendix table 3 and figure 6.

Figure 6. Growth Curve of Viable Cells Without Medium Change



Establishment of normal growth curve. A normal growth curve of cultured diploid mammalian cells consists of an initial lag phase followed by a logarithmic growth phase which reaches a point of maximum growth before going to a logarithmic decline. In this investigation, one objective was to observe if any change in the growth pattern of the cells occurs after having been cultivated over a long period of time. Cells of an early passage, P-276, were used for the first determination. Appendix table 4 and figure 7 show that a sharp decrease in cell number took place from inoculation to 24 hours. The number of non-viable cells, however, was not excessively high. This initial decrease did not occur in the cells from the older passage, P-296. These older cells established a curve similar to diploid cultured cells. A steady rise in viable cell number was noted until a peak was reached at 48 hours. A slight decrease took place in the number of viable cells by the 72 hour point.

Views of cells growing under normal culture conditions, in the polystyrene flasks, are shown in figure 8 under both low and high magnification. A portion of a hemocytometer field is shown in figure 9. This view of stained and unstained cells is typical of what was observed during the investigations.

Controls. For the major portion of this investigation, another normal growth curve was made. No cell count was available for 24 hours following the initial subculture since this was the 0 hour treatment time. The control, however, did exhibit a rise in viable cell number from the inoculation level to the point 24 hours after treatment (the time equivalent to 48 hours after subculture). A decrease in cell number within the next 24 hour period, similar to the drop noted in the 48 to 72 hour

Figure 7. Normal Growth Curves

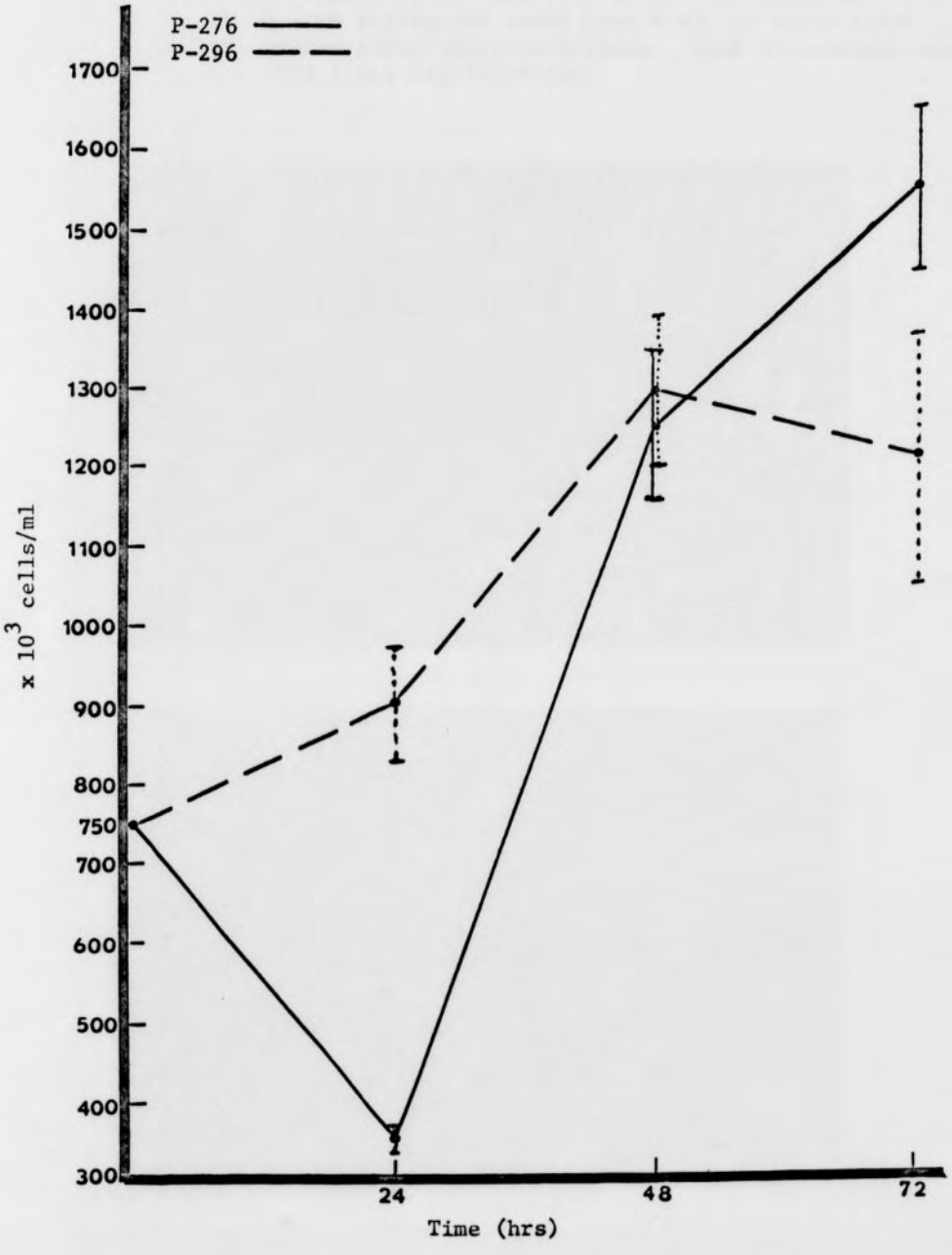


Figure 8. Black and White Photograph of Cells in Culture
A. Top photograph shows cells under low power growing in Falcon polystyrene flasks under phase conditions. 100X microscopic and 370X total magnification.
B. Bottom photograph shows same field of cells under higher power phase conditions. 200X microscopic and 550X total magnification.

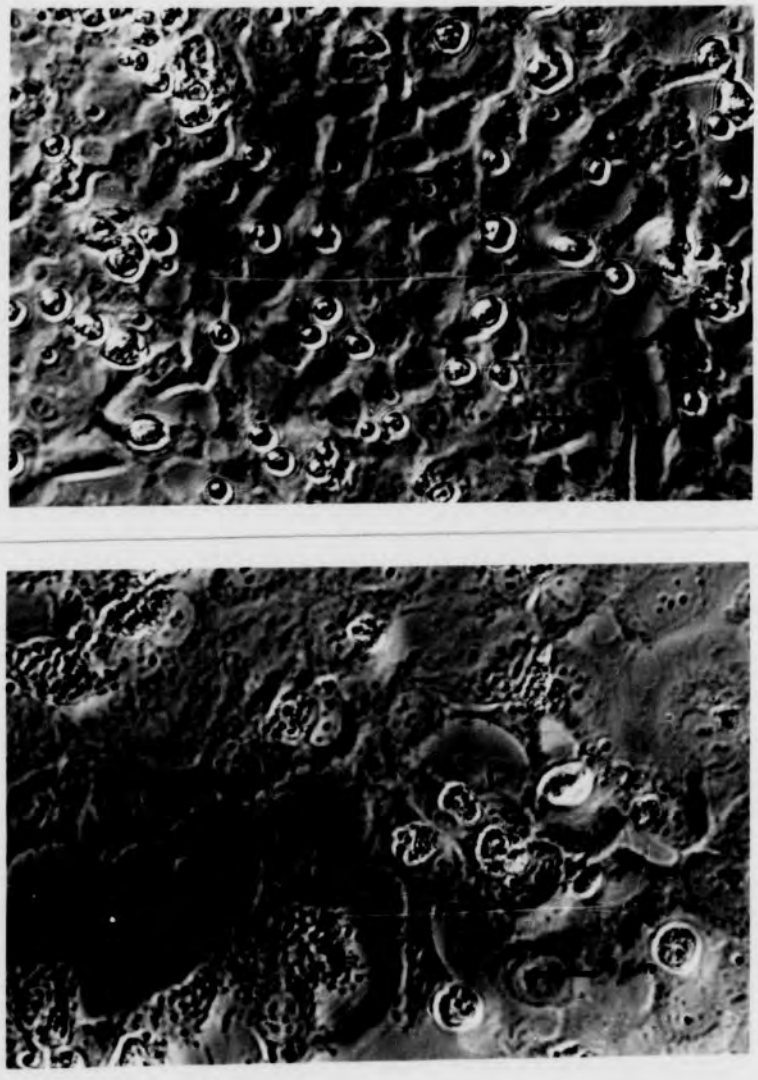
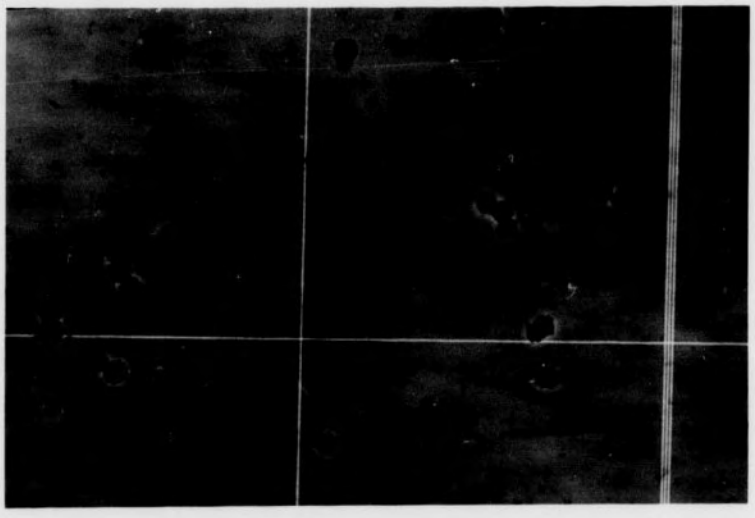


Figure 9. Hemocytometer Field

Note stained non-viable and unstained viable cells.
100X microscopic and 370X total magnification.



period for the P-296 normal growth curve, also occurred. A count of the cells removed in the supernatant at this time, however, was approximately 336.6 x 10^3 cells/ml. If these cells had remained attached, a rise in the curve would have been observed. The curve shows a decline in the next time period; and even if the 393.8 x 10^3 cells/ml counted in the supernatant had remained attached, a decline would still have occurred. The number of non-viable cells counted for each time period also increased steadily with time. Exact data is presented in Appendix table 5 and in figure 10. A control also carried out with a level of 95% ethanol equivalent to that used for the vitamin A dilution (0.25% ethanol) showed a slight increase in cell number within the 24 hour period, followed by steady decreases. The number of cells taken off with the supernatant, 268.4 x 10^3 cells/ml at 48 hours and 523.6 x 10^3 cells/ml at 72 hours after treatment, indicated that cell proliferation was still taking place. A statistical comparison was made between the means of the control and alcohol control tests, and the only time period in which any statistically significant difference was observed was at 48 hours (Appendix table 6).

Progynon estradiol. The cells treated with a therapeutic source of 17 β-estradiol, Progynon, exhibited unusual reactions. A decrease in the number of viable cells/ml was observed. Appendix table 5 and figure 11 show the first cell count made 24 hours after the 1 hour exposure (48 hours after subculture) dropped lower than the inoculation level. There was a small decrease in the next time period; although the cell count made of the supernatant, 330.0 x 10^3 cells/ml would have raised the number of cells above the initial level. The count of the cells still attached to the surface by the last time period was again greatly reduced. If the

Figure 10. Growth Curves: Untreated Control, Alcohol Control

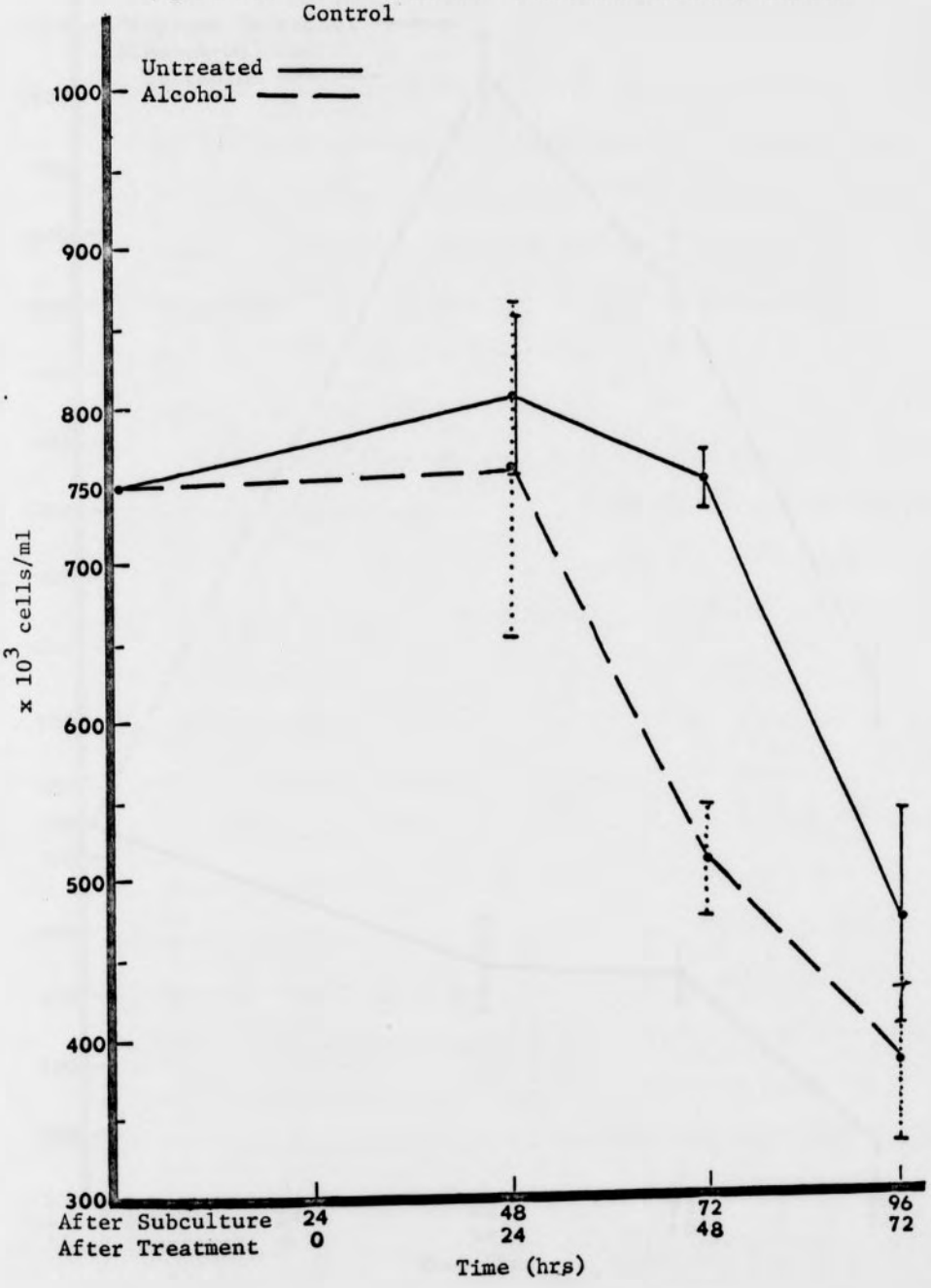
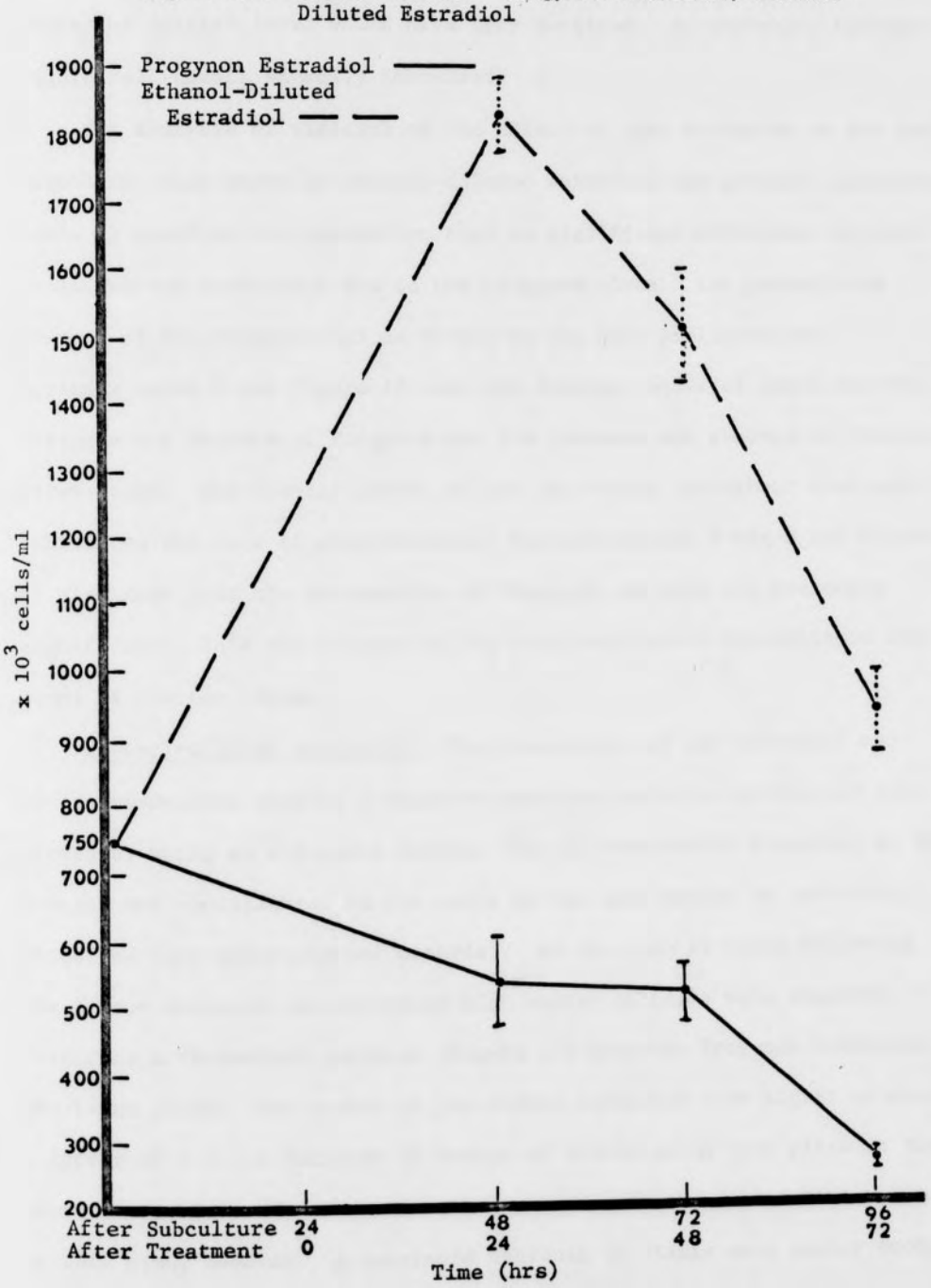


Figure 11. Growth Curves: Progynon Estradiol, Ethanol-Diluted Estradiol

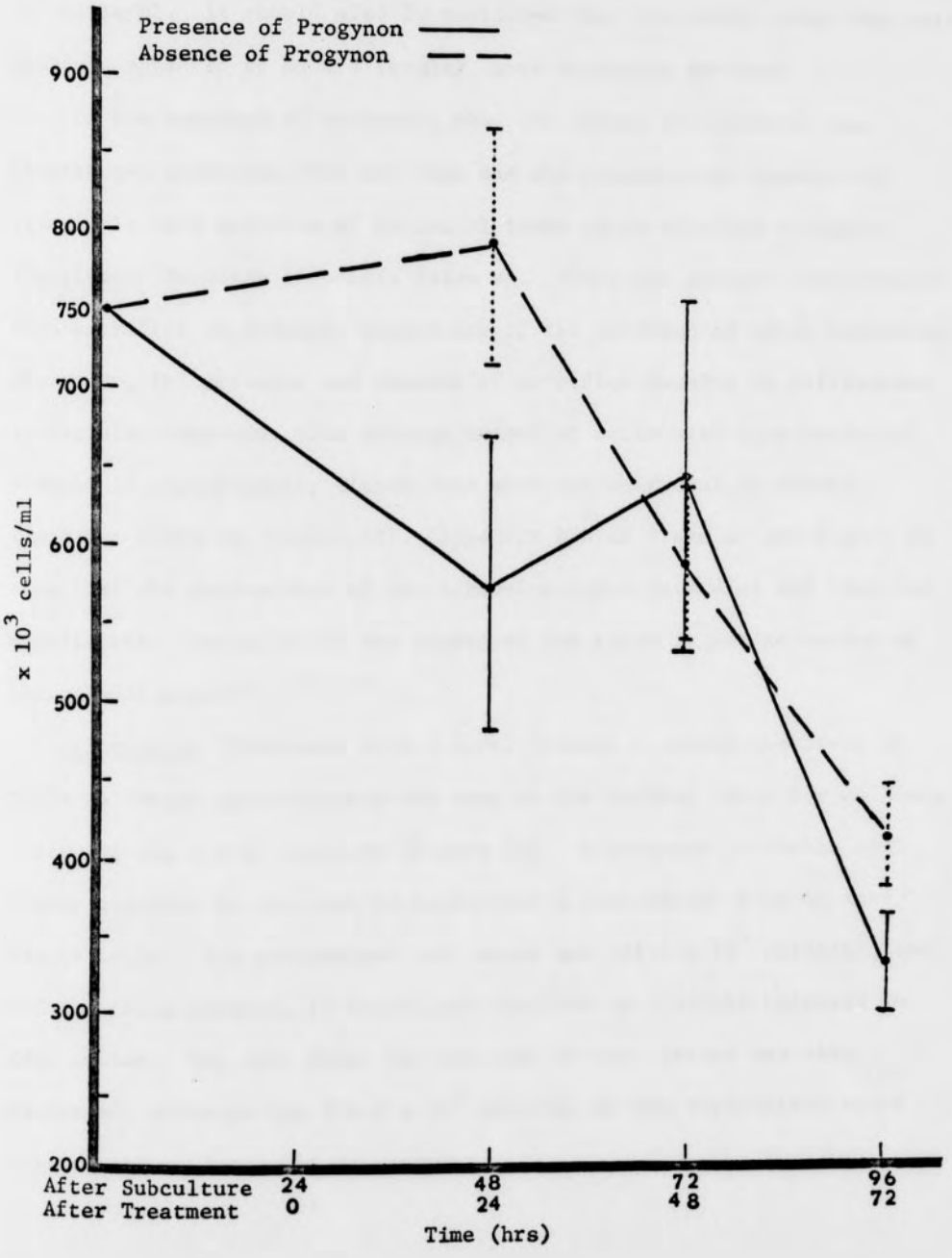


cells in the supernatant had remained attached, a level just slightly above the initial level would have been obtained. As expected, the non-viable cell counts steadily increased.

The analysis of variance on the effect of test variables on the cell growth in cases where no ethanol-diluted estradiol was present, (Appendix table 7) verifies the assumption that no significant difference in cell proliferation took place due to the Progynon alone. The presence or absence of the Progynon had no effect on the cell proliferation. Appendix table 8 and figure 12 show the average number of cells for the presence and absence of Progynon and the presence and absence of Progynon versus time. The similar levels of the two curves throughout time substantiates the lack of significance. Appendix tables 7 and 8 and figure 12 also show that the interaction of Progynon and time was not quite significant. This can be seen by the relative lack of variation in the slope of the two lines.

Ethanol-diluted estradiol. The possibility of the estradiol molecules themselves causing a negative reaction could be verified or disproved by using an alternate source. The 17 β-estradiol dissolved in 95% ethanol was administered to the cells in the same manner as previously described (see materials and methods). At the time 24 hours following the 1 hour exposure, an extremely high number of cells were observed. More than a three-fold increase (figure 11) over the Progynon treatment had taken place. The number of non-viable cells was also higher by about a factor of 4.7. A decrease in number of viable cells took place in the next time period. No count of cells removed with the supernatant was made at this time, however. A continued decrease in viable cell number took

Figure 12. Interaction of Progynon Estradiol and Time



place by 72 hours following treatment, and the non-viable cell count also increased. A supernatant count for this final time period was 1247.4 x 10^3 cells/ml. It should also be mentioned that the cells, when they were counted, appeared to have a rougher, more irregular surface.

In the analysis of variance, when the effect of estradiol was considered, averaging over all time and the presence and absence of vitamin A, this solution of estradiol taken alone elicited a highly significant response (Appendix table 9). There are greater cell numbers when estradiol is present, regardless of the addition of other variables; therefore, the presence and absence of estradiol results in differences in cellular response. The average number of cells with this estradiol present is significantly higher than when the estradiol is absent (Appendix table 10, figure 13). Appendix tables 9 and 10 and figure 13 show that the interaction of the ethanol-diluted estradiol and time was significant. Variation in the slopes of the lines is an indication of this significance.

Vitamin A. Treatment with 5 µg/ml vitamin A caused the level of cells to remain approximately the same as the initial level for 24 hours following the 1 hour exposure (figure 14). A decrease in viable cell number occurred in the next 24 hours with a concomitant rise in non-viable cells. The supernatant cell count was 378.4 x 10^3 cells/ml; and had the cells altered, it would have resulted in a slight increase in cell number. The cell count for the next 24 hour period was also decreased, although the 594.0 x 10^3 cells/ml in the supernatant would have caused an increased cell number.

Figure 13. Interaction of Ethanol-Diluted Estradiol and Time

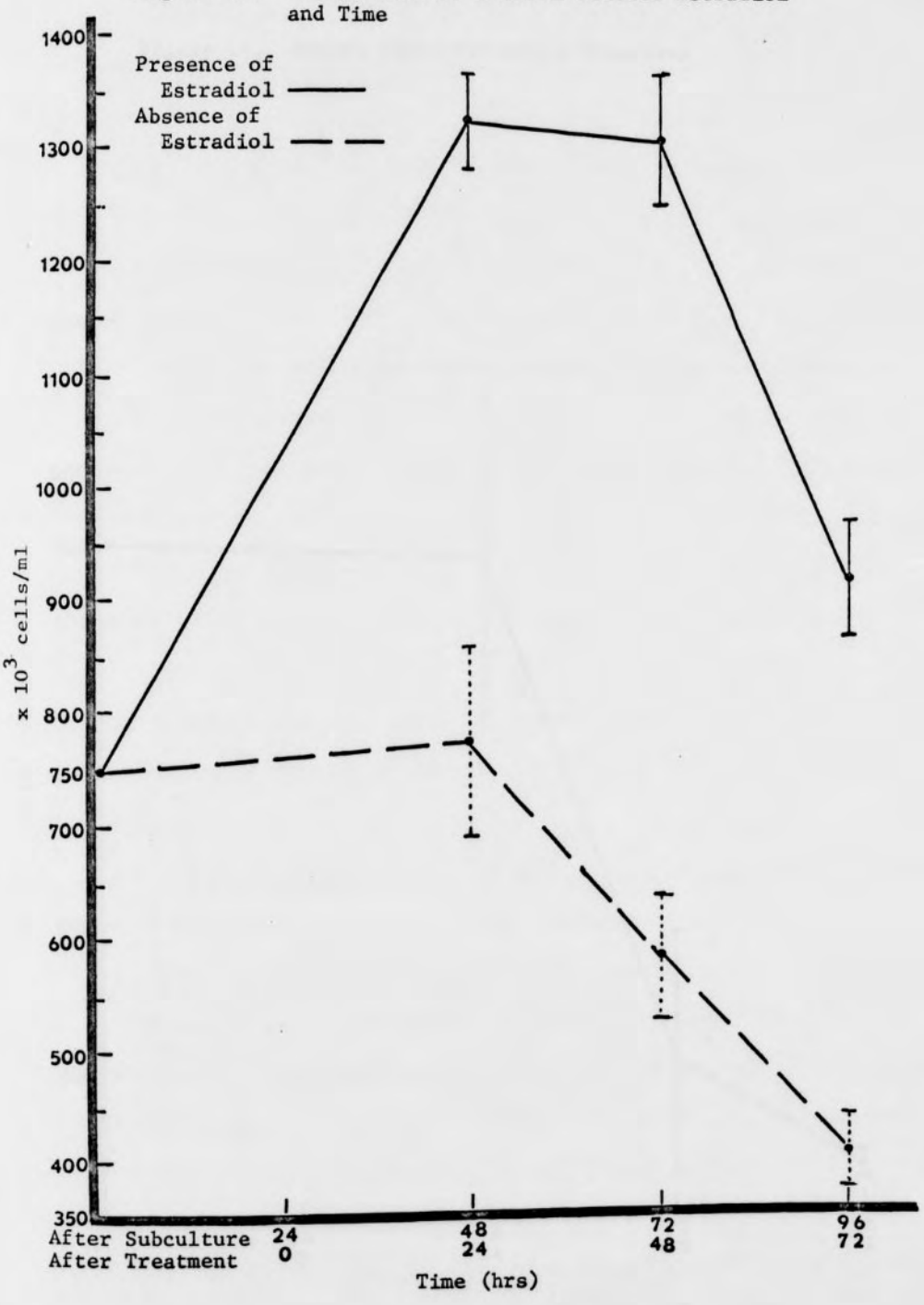
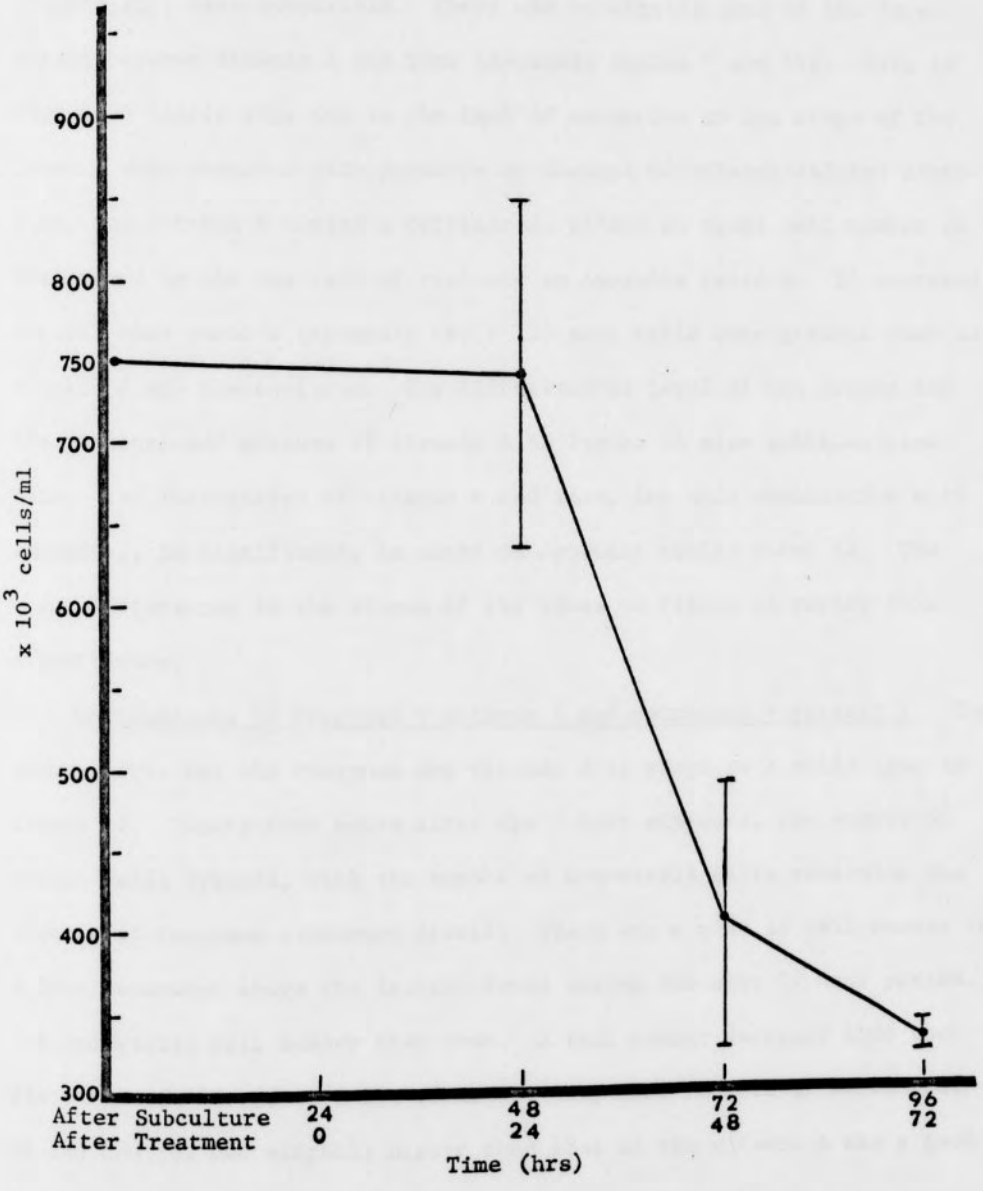


Figure 14. Growth Curve-Vitamin A Treatment



A statistical comparison of the presence and absence of Progynon showed no significance in cell proliferation due to the presence or absence of vitamin A (Appendix tables 7 and 11). The levels of the graphs for presence and absence of vitamin A, when only Progynon was used (figure 15), were comparable. There was no significance in the interaction between vitamin A and time (Appendix tables 7 and 11). Data in figure 15 verify this due to the lack of variation in the slope of the lines. When compared with presence or absence of ethanol-diluted estradiol, the vitamin A caused a detrimental effect on total cell number as determined by the analysis of variance in Appendix table 9. If averaged for all time periods (Appendix table 12) more cells were present when no vitamin A was administered. The difference in level of the graphs for the presence and absence of vitamin A in figure 16 also substantiates this. The interaction of vitamin A and time, for this combination with estradiol, is significant, as noted in Appendix tables 9 and 12. The sharp differences in the slopes of the lines in figure 16 verify this significance.

Combinations of Progynon + vitamin A and estradiol + vitamin A. The growth curve for the Progynon and vitamin A is shown as a solid line in figure 17. Twenty-four hours after the 1 hour exposure, the number of viable cells dropped, with the number of non-viable cells remaining the same as in Progynon treatment itself. There was a rise in cell number to a level somewhat above the initial level during the next 24 hour period. The non-viable cell number also rose. A cell number decrease then took place between 48 and 72 hours after the treatment to a level below that of the control but slightly higher than that of the vitamin A and a good

Figure 15. Interaction of Vitamin A and Time in Absence of Ethanol-Diluted Estradiol

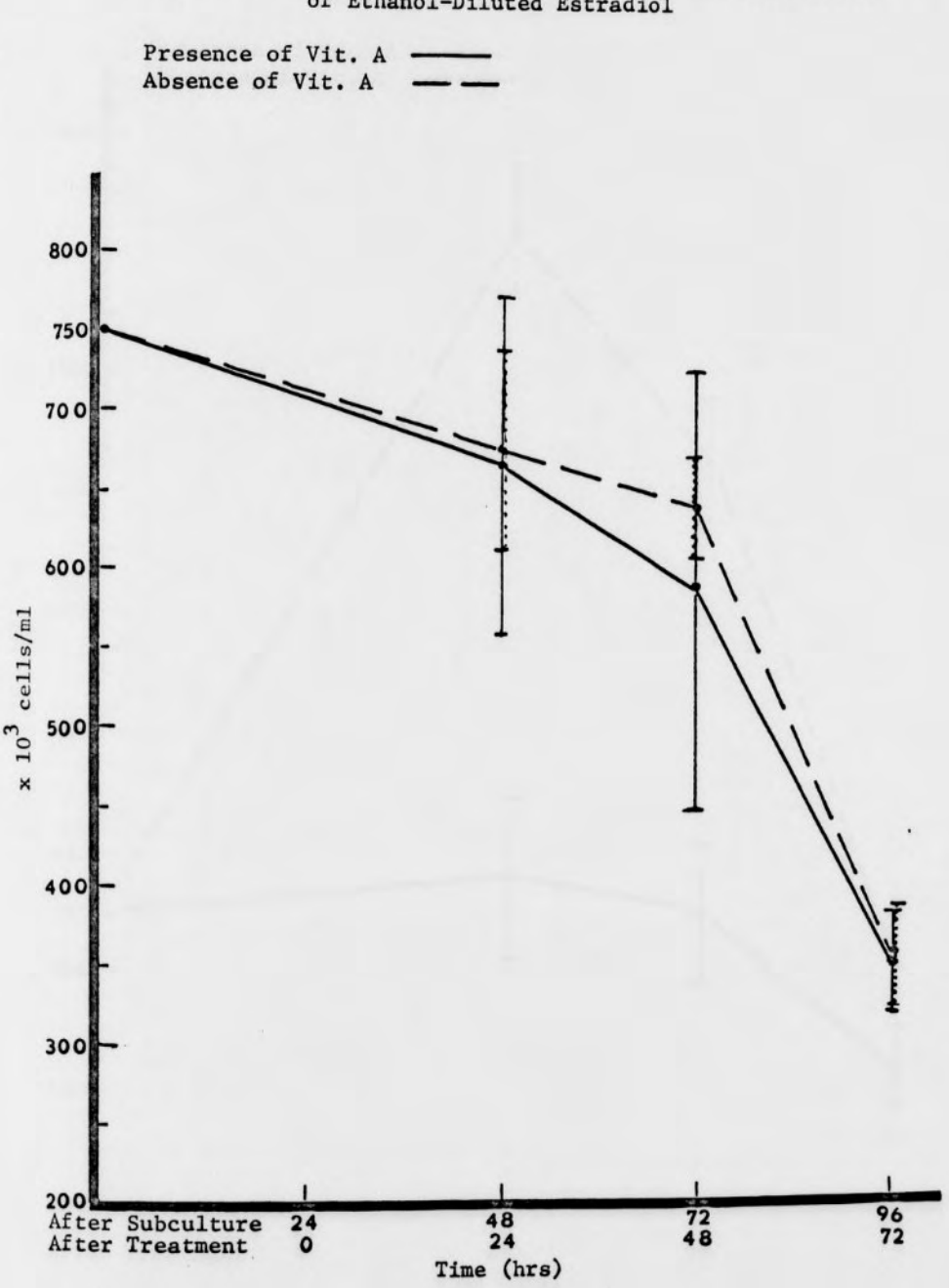


Figure 16. Interaction of Vitamin A and Time in Absence of Progynon Estradiol

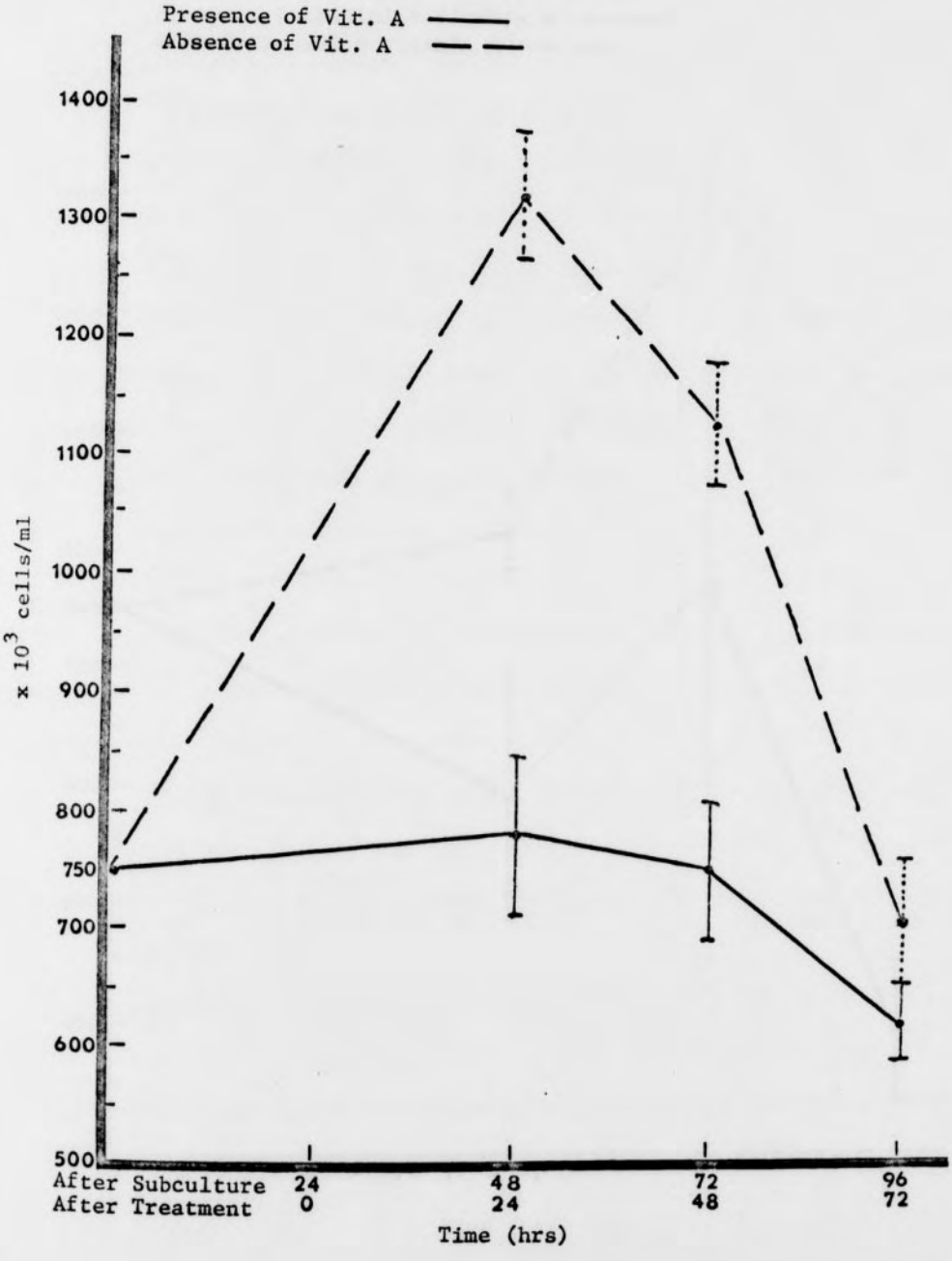
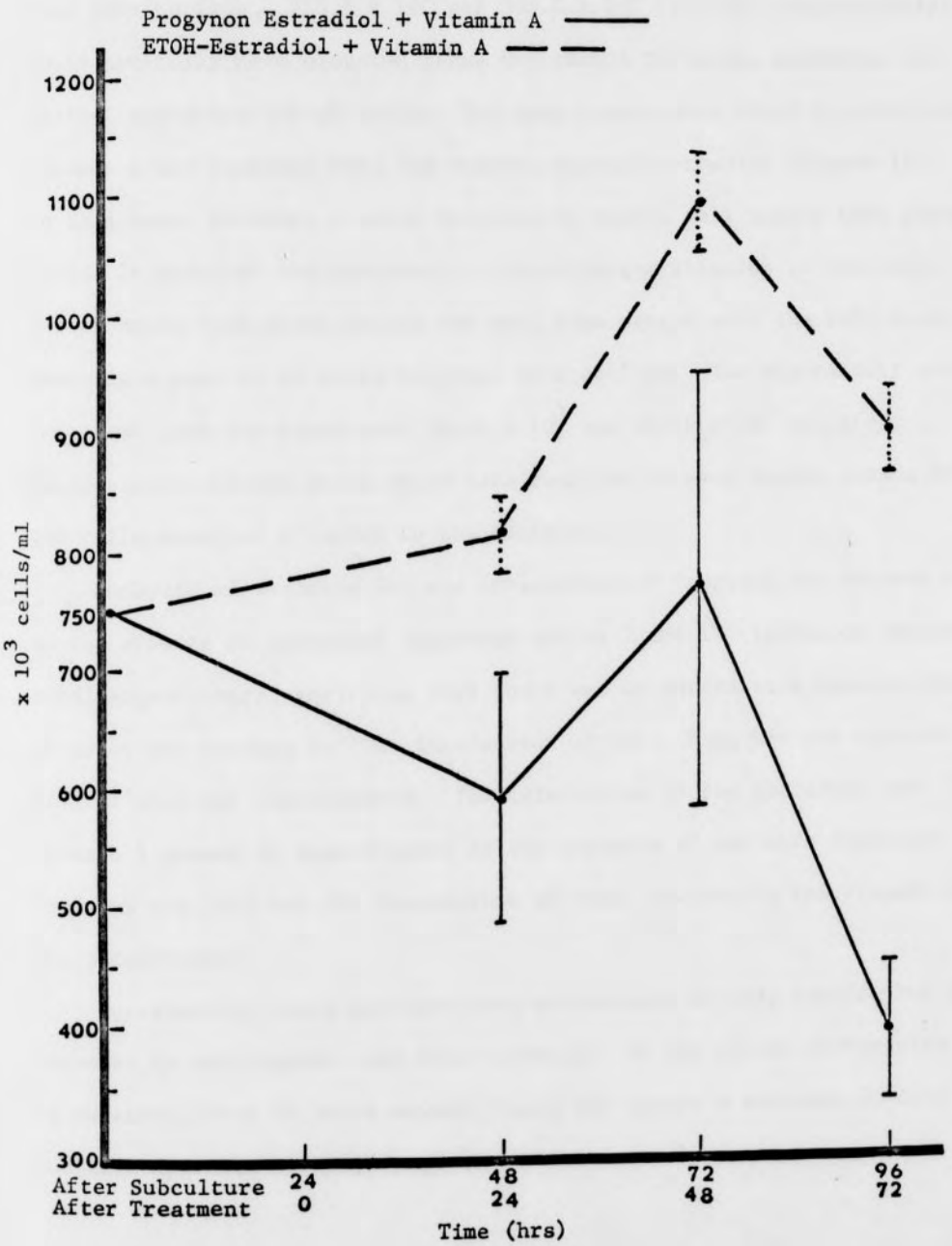


Figure 17. Growth Curves: Progynon Estradiol + Vitamin A and Ethanol-Diluted Estradiol + Vitamin A



deal higher than the Progynon treatments. The non-viable count was lower than at the previous time. Supernatant cell counts for the 48 and 72 hour time periods, 358.6×10^3 and 539.0×10^3 cells/ml, respectively, would naturally have elevated these two counts to levels exceeding the initial concentration of cells. The same trends were found to occur when vitamin A was combined with the ethanol-diluted estradiol (figure 17). In this case, however, a small increase in viable cell number took place within 24 hours of the treatment. A much larger stimulus to the cell growth again took place within the next time period with the cell count reaching a peak at 48 hours followed by a decline. The supernatant counts for these last two times were 101.2×10^3 and 347.6×10^3 cells/ml, respectively, levels which would have resulted in even higher counts had the cells remained attached to the surfaces.

Analysis of variance for the interaction of Progynon and vitamin A in the absence of estradiol (Appendix tables 7 and 13) indicated statistical significance, verifying that there was an interaction between the Progynon and vitamin A. The interaction of time, Progynon and vitamin A did not show any significance. The interaction of the estradiol and vitamin A showed no significance in the analysis of variance (Appendix tables 9 and 14); but the interaction of time, estradiol, and vitamin A was significant.

Occasionally there may have been an increase in cell number, but a decrease in cell number took place overall. If all growth curves were to be examined, from 24 hours onward, there was always a decrease in cell number. Appendix tables 15 and 16 verify these observations.

Chromosomes. Information supplied by the American Type Culture Collection on I-407 indicated a modal chromosome number of 76. In the brief chromosome investigation that was carried out, an average number of 77.5 chromosomes per cell was obtained. Several unusual or marker chromosomes were detected as well as what appeared to be chromosome pieces. A typical chromosome spread and karyotype may be seen in figures 18 and 19, respectively. No complete statistical determinations of chromosome numbers were made since the purpose was simply to develop the technique for chromosome examinations and possibly provide a rough confirmation of previous information of aneuploidy. Aneuploidy was confirmed in part by the finding of unusual nuclei and mitotic figures (figure 20). An unusual mitotic figure, what has been termed a "trefoil" in this laboratory due to its cloverleaf appearance, may be seen in figure 21.

Figure 18. Typical Chromosome Spread of Cells in Culture

Figure 19. Karyotype of Chromosome Spread

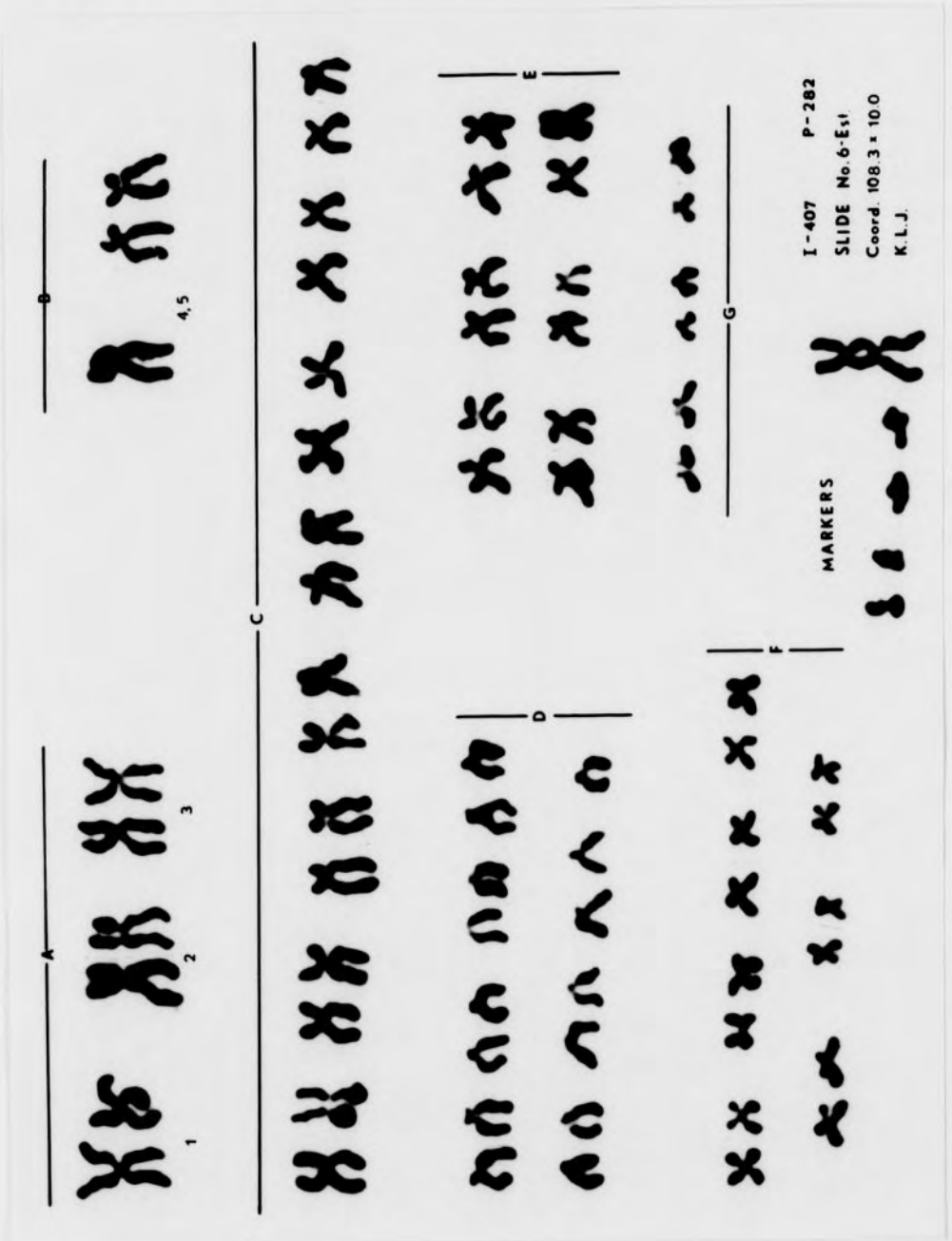


Figure 20. Two Color Photographs Displaying Aneuploid Nuclei

Note other stages of mitosis. Cell cycle stain used. 400X microscopic and 740X total magnification.

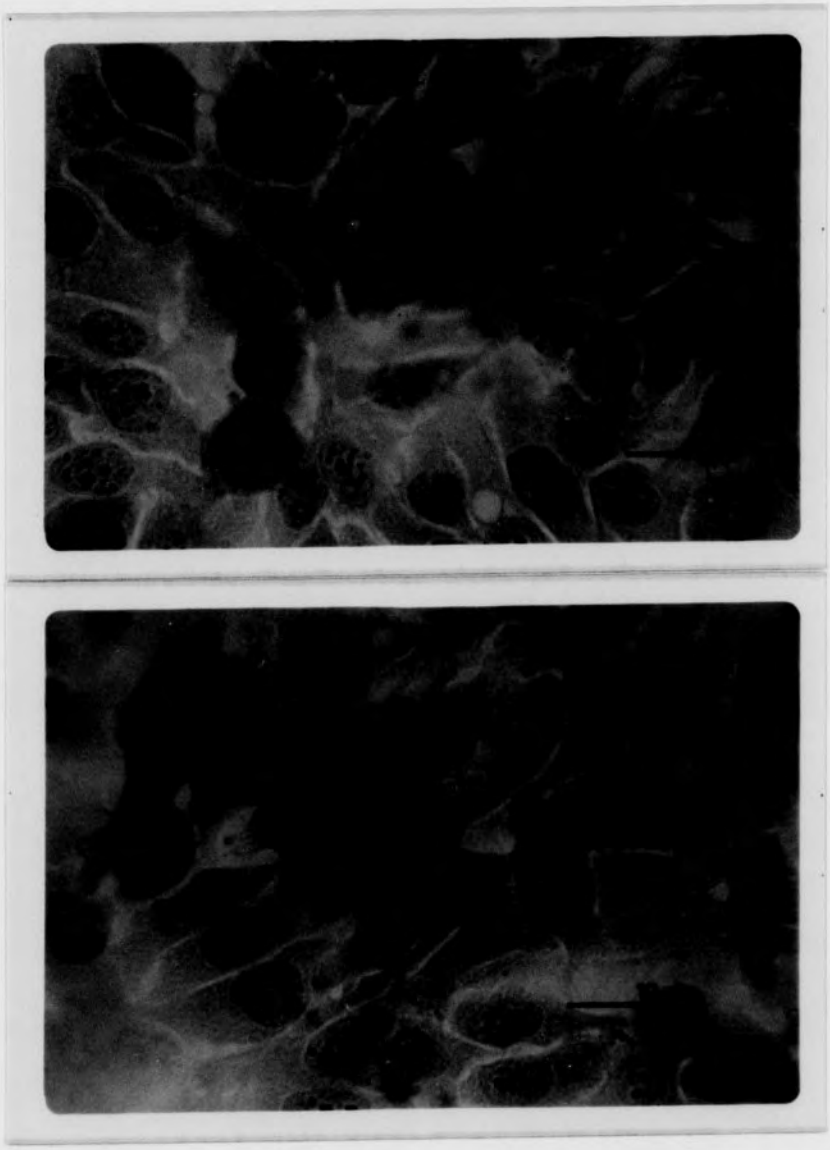
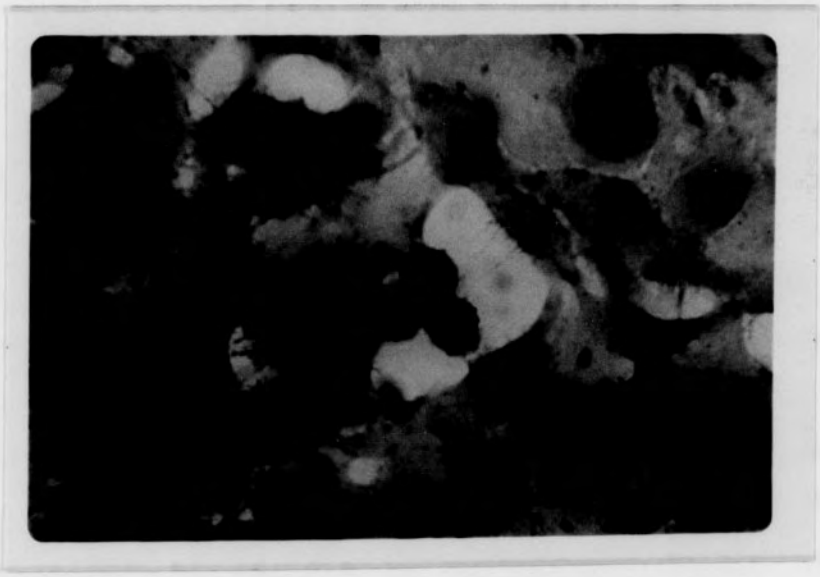


Figure 21. Color Photograph of "Trefoil" Mitotic Figure

Lehman's polychrome stain used. 400X microscopic and 740X total magnification.



CHAPTER V

DISCUSSION

Although different experimental conditions existed in the preliminary and major experiments, the data from the preliminary experiments was not pertinent to the final conclusions; therefore, it is not included in this discussion. Results from each portion of the major experiments will be discussed separately.

No medium change. When the culture medium was not changed the day following subculture, the critical point of cell proliferation occurred at 48 hours. Three possible explanations may be suggested for the sharp decline in viable cells which took place after the 48 hour point: 1). Waste materials and cellular debris remaining from the subculturing procedure may exert a toxic effect on the cells, thus causing cell death. 2). Some essential growth factor, such as the one in calf serum mentioned by Temin, et al. (1972), may be depleted. 3). A combination of these two possibilities may also exist. Small amounts of toxic substances may accumulate but do not exert significant damage until the cells are weakened by the depletion of another essential substance or substances. Extensive biochemical assays, both before and after growth of the cells in the medium, would be required to determine existence of toxic substances or absence of an essential growth factor.

Establishment of normal growth curve. The first point to be considered in establishing growth curves was the sharp decrease in cell number which took place in the P-276 cells from the time of inoculation

to 24 hours. Since the non-viable cell count for the period under consideration was not excessively high, it would seem improbable that a latent virus had been induced to become lytic. Had this been the case, the lytic or cytopathic effect would have been detected throughout the remainder of the samples. A more plausible explanation would be that some substance essential for cell attachment and proliferation was lacking or inactive following subculture. Medium change at the 24 hour point provided fresh resources of the substance and stimulated cell proliferation. Both young (P-276) and old (P-296) cells exhibited the same important increase in growth between 24 and 48 hours. The growth curve of the P-276 cells continued to rise after 48 hours. It would be desirable to know what these cells would have done after 72 hours - if this was the peak of growth or if the peak occurred at a later time. Further replications should also be made to confirm the decrease in the growth curve of the older cells after 48 hours. This information would be useful when planning further experiments since it would be desired to use cells in the period of growth just before the peak.

Controls. The growth curve of cells treated with alcohol (95% ethanol) was at a lower level than the untreated control. The only point of significant difference was at 48 hours after treatment. The large number of viable cells recovered in the supernatant after subjection to alcohol indicated that cell proliferation was still taking place. The alcohol molecules might prevent the cells from attaching to or remaining attached to the culture surface. Cells in mitosis tend to round up and decrease cell attachment areas. One speculation might be that the alcohol molecule reacts in some degradative way with proteins holding

the cells down or that formation of the attachment proteins is repressed. More extensive research would be necessary for determining precise causes for these cellular reactions.

Progynon estradiol. The obvious question which arises concerning the cells receiving the Progynon estradiol is what caused the steady death of the cells? The most likely answer to this is that the Progynon estradiol suspension contained small amounts of polysorbate 80 and phenol which caused cell death. Individual tests would be required to determine which one of these substances cause the harmful effects or if the two act synergistically to damage the cells. It is possible that these substances or the 17 β-estradiol molecules, as suggested by Szego (1972), could damage lysosomal membranes causing cellular autolysis. If so, or if a latent virus had been induced, a greater number of non-viable cells would have been present in the supernatant. There is a distinct difference in *in vitro* and *in vivo* conditions with reference to cell attachment. In light of this, the genomes of the cells may be prohibited from forming attachment proteins and possibly caused to slow down or stop the replication process. While the Progynon solution has been used for *in vivo* experiments (Sansone-Bazzano, et al., 1972) and therapeutically, no *in vitro* work reported in the literature reviewed made use of the solution. The filtration by the liver and other body components when injected into the body might eliminate any harmful effects by the preservatives in the estradiol solution. It is recommended that this form of 17 β-estradiol not be used for *in vitro* experimentation.

Ethanol-diluted estradiol. Cells exposed to this estradiol elicited an initial surge of growth followed by a severe decrease in cell number

after the 24 hour point. It was not surprising to find this decrease in number of cells remaining attached to the surface after the initial surge. Available surface area was evidently depleted, and the cells were forced off. Such sloughing off of overcrowded surfaces is a familiar occurrence to tissue culture investigators. It was also observed in this instance that many cells were packed together due to lack of space and somewhat rounded up at later times after treatment. Consequently there were a great number of cells floating in the supernatant. The lack of room on the surface for growth and the vast number of cells depleting the nutrients required for growth are attributed as causes for increased numbers of non-viable cells. As Szego suggested (1972), estradiol molecules may have destabilized lysosomal membranes enough to allow release of lysosomal enzymes, thus providing an explanation for higher numbers of non-viable cells. Further biochemical and histochemical studies would be required to verify this latter postulation. It is recommended that this solution of 17 β-estradiol be used for in vitro investigations. Adequate space must be provided, however, for the cells to spread out if experiments include any lengthy incubation following treatment.

Vitamin A. The observations of high numbers of viable cells in the supernatant and decreasing numbers remaining attached to the surface after vitamin A treatment suggested that something prevented the dividing cells from attaching and spreading out on the surface. It has been stated (Szego, 1972; Willmer, 1970) that vitamin A may exert a destabilizing effect on lysosomal membranes. If this had taken place in these experiments, it would have seemed that the non-viable cell count, both from the surface and the supernatant, would have been higher. Although no

morphological changes could be distinguished in the counts due to the action of vitamin A, it is possible that some such changes were taking place. Enzyme activities may also have been altered or modified, as well as changes or increases in the membrane permeability of the cells, thus accounting for some of the results. Since exact levels of vitamin A to be used for optimum *in vitro* action were difficult to determine, it is highly possible that a toxic level of vitamin A was used and was the cause of cell death. Testing various levels of vitamin A for optimum cellular response is strongly recommended before further *in vitro* research with vitamin A is performed.

Combinations of Progynon + vitamin A and estradiol + vitamin A. The treatment of the cells with the Progynon estradiol plus vitamin A will be discussed first. After an initial drop in cell number, the cells exhibited a small spurt of growth or proliferation to reach a peak 48 hours after treatment. Since Progynon alone continued in a downward trend throughout the testing periods, there must be some synergistic effect of Progynon and vitamin A building up and causing a stimulus to the cells between the 24 and 48 hour times. When ethanol-diluted estradiol was combined with vitamin A, a small rise in cell number took place within the first 24 hours after treatment with a peak of proliferation again being reached at 48 hours. This initial increase was evidently due to the action of the estradiol. Vitamin A, it may be speculated, must have suppressed some of the potential estradiol action since the cells did not proliferate to as great a degree. Several hypotheses may be drawn to explain these results: 1). It may be that the vitamin A altered cell permeability to such an extent that the estradiol could not penetrate as

effectively. An allosteric effect in terms of altering receptor site configuration might be caused by the vitamin A, thus preventing as many cytoplasmic receptor sites from picking up and transporting estradiol molecules to the nuclear receptors. 2). Perhaps molecules of vitamin A and estradiol interact or form a complex which takes longer, up to 24 to 48 hours, to elicit a reaction by the replicative machinery of the cells. 3). Vitamin A may cause some changes in the cellular enzymes that would in some way hinder the translocation of the estradiol-receptor complex to the nucleus. Since this translocation is temperature-dependent (Jensen and DeSombre, 1973), it could be dependent on some cytoplasmic enzymes or structures which might be altered by the vitamin A. 4). The fact that both substances are known to destabilize lysosomal membranes presents another problem. Since the amount of cell death detected did not exceed that which occurred with the individual tests of the substances, no destabilization could have occurred. The same holds true for possible virus induction. While it is known that many established cell lines or cells grown in tissue culture do carry latent viruses which may at some time exhibit cytopathic effects, it does not appear from these results that any viruses are being induced. This question has been raised in the past, however, when no ready or detectable reason for failure of cultures to survive could be determined. Viruses are entities of which tissue culture technicians must be constantly wary.

Chromosomes. As previously noted, the I-407 cells were aneuploid. Normal diploid human cells contain 46 chromosomes, but the average number counted in the cultured cells was 77.5. Several unusual chromosomes and chromosome pieces were also found. Fusion of nuclei, partial failure of

cytokinesis after karyokinesis, or irregular chromosomal replication may account for the aneuploidy. Established cell lines are, however, distinguished by their transformed, aneuploid state.

CHAPTER VI

SUMMARY AND RECOMMENDATIONS

Summary

1. Cultured intestinal epithelial cells (I-407) and standard tissue culture techniques were used to study the following: a) the necessity of changing culture medium the day following subculture; b) the normal growth curve of an early and older passage of cells; c) the alterations in the growth curve of the cells after a 1 hour exposure to 17 β-estradiol, vitamin A, estradiol-vitamin A combination, alcohol (95% ethanol) control, and untreated control; d) differences in cellular response to therapeutic and ethanol-diluted sources of 17 β-estradiol; and e) a method for observing chromosomal variations after various passages.

2. Preliminary experiments involved growth of cells on coverslips in Leighton tubes, treatment with Progynon estradiol, vitamin A, estradiol-vitamin A combination, and control for as long as 48 hours, and the determination of the mean number of total cells per slide after staining. Progynon estradiol treatment exhibited a slight increase over the inoculation level and an irregular growth pattern. Compared to the control, cells proliferated more slowly and variably after vitamin A treatment, and more variations in growth took place after the combination treatment.

3. A system of determining the number of viable and non-viable cells/ml in test samples was adapted from a technique developed by Yip and Auersperg (1972) and tested for the cells in use.

4. Techniques for measurement of cell growth were reviewed, and hemocytometer cell-count procedures for I-407 cells were established.

5. The concentration of 17 β-estradiol chosen for this study was based on the concentration found in the blood of adult females. The 0.02 µg/ml level is an intermediate level of estradiol present during pregnancy (0.015 to 0.030 µg/ml) and represents the concentration added to the bloodstream by a 2 mg birth control pill. This concentration of 0.02 µg/ml 17 β-estradiol was found to increase proliferation of I-407 cells in vitro.

6. The choice of vitamin A concentration for the investigation was based on reported determinations of vitamin A concentration in human tissues (Raica, et al., 1972). An approximate level of 1 µg/ml vitamin A in tissues other than the liver was found, and other experimentation in vitro suggested an optimum concentration of 5 µg/ml. This concentration of vitamin was chosen and was found to inhibit cell proliferation.

7. A critical point after subculture of cells was found to be at 48 hours if culture medium was not replaced after the subculture. Thus a medium change is necessitated within 48 hours after subculture.

8. Growth curves of cells from young (P-276) and old (P-296) cells were determined from cultivations and counts for 24, 48, and 72 hours. The cells in P-276 continued to exhibit an upward trend in growth through 72 hours, and the P-296 cells appeared to reach a peak at 48 hours.

9. Cell counts were made 24, 48, and 72 hours after a 1 hour exposure to untreated control, alcohol (ethanol) control, Progynon estradiol, ethanol-diluted estradiol, vitamin A, and the estradiol-vitamin A combinations. The ethanol appeared to inhibit cell growth, although the

only significant difference between the two controls was at 48 hours. Progynon estradiol exerted a deleterious effect, whereas the cell numbers increased greatly following treatment with the ethanol-diluted estradiol. Vitamin A also inhibited cell proliferation and appeared to interact with the stimultaneous administration of Progynon but not ethanol-diluted estradiol.

10. A general estimate of the cellular chromosome number confirmed previous information of aneuploidy of the cultured cells.

Recommendations

1. It is recommended that dose response investigations be conducted with estradiol and vitamin A to determine optimum concentrations for *in vitro* work.

2. The use of the ethanol-diluted source of 17 β-estradiol is recommended for further experimentation *in vitro*.

3. Since these cells proved to be responsive to the estradiol, it is suggested that an investigation be carried out to determine the presence and number of receptor molecules per cell.

4. The choice of different time intervals for examination (ie. 4, 8, 12 hours, etc.) would provide more exact times of action of the substances.

5. More detailed chromosome analyses should be done for more exact identification of the chromosomes in groups and to determine any variations after different passages. Labeled estradiol may be used for locating loci of estradiol action on the chromosomes.

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APPENDIX

TABLE 1

STAINING PROPERTIES OF I-407 CELLS: TIME VARIATIONS AFTER GLUTARALDEHYDE FIXATION
(Mean Cell Numbers)

Time after fixation	Viable Cells/ml$\times 10^3$	Non-viable Cells/ml$\times 10^3$
1 hour	215.67 ± 4.37	12.67 ± 0.67
24 hours	227.33 ± 22.88	16.67 ± 4.98
48 hours	183.67 ± 9.21	16.67 ± 2.67
72 hours	198.00 ± 14.29	16.00 ± 1.53

± standard error of mean values

NOTE: The close correlation of the numbers of non-viable cells after 24, 48, and 72 hours after fixation justifies procedure of making cell counts between 24 and 72 hours after fixation.

TABLE 2

PRELIMINARY EXPERIMENTAL DATA
MEAN TOTAL CELL NUMBERS ± S. E.

Time of Treatment	Control	Progynon Estradiol	Vitamin A	Estradiol + Vitamin A
0 hr.	4.79			
24 hrs.	32.09 ±3.41	20.13 ±1.04	15.80 ±1.91	34.05 ±4.94
48 hrs.	59.27 ±9.94	47.18 ±7.02	57.04 ±11.79	22.77 ±1.52
72 hrs.	69.84 ±9.54	38.55 ±2.22	49.64 ±4.75	63.09 ±5.29
96 hrs.	79.91 ±8.31	74.84 ±12.23	88.85 ±10.83	69.96 ±10.27

TABLE 3

VIABLE AND NON-VIABLE CELLS/ml x 10^3:
NO MEDIUM CHANGE FOLLOWING SUBCULTURE

Time of Growth	Viable Cells	Non-Viable Cells
24 hours	955.99 ±74.27	23.47 ±6.01
48 hours	1214.41 ±101.81	27.13 ±1.81
72 hours	781.73 ±69.33	72.67 ±3.67

± standard error of mean values

TABLE 4

VIABLE AND NON-VIABLE CELLS/ml x 10^3:
ESTABLISHMENT OF NORMAL GROWTH CURVE

P-276 Time of Growth	Viable Cells	Non-Viable Cells
24 hours	358.36 ±23.80	26.40 ±0.85
48 hours	1266.95 ±97.40	55.49 ±9.29
72 hours	1556.38 ±110.93	81.40 ±8.33

P-296 Time of Growth	Viable Cells	Non-Viable Cells
24 hours	907.13 ±71.18	16.13 ±4.81
48 hours	1297.71 ±95.82	23.98 ±1.46
72 hours	1221.95 ±162.20	13.71 ±2.50

± standard error of mean values

TABLE 5

VIABLE AND NON-VIABLE CELLS/ml x 10^3 FOLLOWING TREATMENT WITH TEST SUBSTANCES

Treatment to cells	Viable Cells			Non-Viable Cells		
	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
Control	809.11 ±55.60	751.91 ±21.93	475.20 ±60.76	14.18 ±2.76	40.82 ±4.40	63.07 ±8.84
Alcohol Control	759.25 ±105.10	501.36 ±35.11	386.47 ±53.54	36.18 ±4.45	40.58 ±8.02	57.93 ±4.04
Progynon Estradiol	533.87 ±76.68	516.02 ±44.62	258.38 ±9.47	22.98 ±6.34	32.02 ±6.59	45.47 ±12.12
Estradiol in ETOH	1825.00 ±62.14	1519.43 ±81.43	927.40 ±66.72	109.02 ±12.63	169.64 ±24.45	289.43 ±53.13
Vitamin A	748.73 ±109.22	407.98 ±86.38	329.75 ±10.92	19.56 ±3.23	29.08 ±8.36	58.92 ±11.36
Progynon + Vitamin A	590.33 ±107.72	762.93 ±192.35	390.38 ±59.86	22.98 ±6.17	47.67 ±6.82	40.09 ±0.88
Estradiol + Vitamin A	817.18 ±27.10	1092.90 ±47.89	903.95 ±43.38	42.29 ±5.12	50.12 ±2.33	96.31 ±5.76

± standard error of mean values

TABLE 6

t-TEST FOR THE SIGNIFICANCE OF THE DIFFERENCE BETWEEN 2 SAMPLE MEANS

t-Test Comparisons	t-Values for: 24 hours	48 hours	72 hours
Control/Alcohol Control	0.4193 n.s.	6.0531***	1.0955 n.s.
Progynon/Control	2.906*	4.744***	3.526*
Estradiol in ETOH/ Control	12.183***	9.113***	5.013***
Estradiol in ETOH/ Progynon	13.082***	10.818***	9.937***
Vit A/Control	0.493 n.s.	3.859**	2.356*
Estradiol in ETOH/ Vit A	8.565***	9.370***	8.849***
Progynon/Vit A	1.610 n.s.	1.111 n.s.	4.937***
Estradiol + Vit A/ Control	0.130 n.s.	6.473***	1.535 n.s.
Estradiol + Vit A/ Estradiol	14.866***	4.519**	0.295 n.s.
Estradiol + Vit A/ Vit A	0.608 n.s.	6.934***	12.836***
Progynon + Vit A/ Progynon	0.427 n.s.	1.250 n.s.	2.178*
Progynon + Vit A/ Vit A	1.033 n.s.	1.683 n.s.	0.996 n.s.
Estradiol + Vit A/ Progynon + Vit A	2.042 n.s.	1.664 n.s.	6.947***

n.s. = not significant
* = $p \leq .05$ (significant)
** = $p \leq .01$ (highly significant)
*** = $p \leq .005$ (very highly significant)

TABLE 7

ANALYSIS OF VARIANCE OF THREE TEST VARIABLES ON CELL PROLIFERATION WITHOUT ETHANOL-DILUTED ESTRADIOL

Source	DF	MS	F
Vitamin A	1	3270.89	1.67
Progynon estradiol	1	55,407.67	0.96
Time	2	317,307.05	14.60*
Progynon x vitamin A	1	242,623.56	5.57*
Progynon x time	2	57,279.36	2.63
Vitamin A x time	2	1968.41	0.09
Vit A x Progynon x time	2	43,586.52	2.01
Error	24	21,730.17	

*indicates significance at the .05 level

TABLE 8

THE EFFECT OF PROGYNON ESTRADIOL PRESENCE IN THE ABSENCE OF THE ETHANOL-DILUTED ESTRADIOL ± S. E.

Time	With Progynon (Progynon, Progynon + Vit.A)	Without Progynon (Control, Vit. A)
24 hrs.	562.10 ± 92.20	778.92 ± 82.41
48 hrs.	639.48 ± 118.49	579.95 ± 54.16
72 hrs.	324.38 ± 34.67	402.48 ± 35.84
Average over time	587.11 ± 88.59	508.65 ± 88.92

TABLE 9

ANALYSIS OF VARIANCE OF THREE TEST VARIABLES ON CELL PROLIFERATION WITHOUT PROGYNON ESTRADIOL

Source	DF	MS	F
Vitamin A	1	1,007,574.29	6.40*
Estradiol	1	3,174,062.93	79.26*
Time	2	489,816.17	42.04*
Estradiol x vitamin A	1	206,134.16	0.85
Estradiol x time	2	40,046.51	3.44*
Vitamin A x time	2	157,410.27	13.51*
Estradiol x vit. A x time	2	241,691.07	20.74*
Error	24	11,650.39	

* indicates significance at the .05 level

TABLE 10

EFFECT OF ESTRADIOL PRESENCE IN ABSENCE OF PROGYNON ESTRADIOL ± S. E.

Time	With Estradiol (Estradiol and estradiol + Vit. A)	Without Estradiol (Control, Vit. A)
24 hrs.	1321.09 ± 44.62	778.92 ± 82.41
48 hrs.	1306.17 ± 64.66	579.95 ± 54.16
72 hrs.	915.68 ± 55.05	402.48 ± 35.84
Average over time	1180.98 ±158.09	587.11 ± 88.59

TABLE 11

EFFECT OF VITAMIN A PRESENCE IN ABSENCE
OF ETHANOL-DILUTED ESTRADIOL ± S. E.

Time	With Vitamin A (Vitamin A, Progynon + Vitamin A)	Without Vitamin A (Control, Progynon)
24 hrs.	669.53 ± 108.47	671.49 ± 66.14
48 hrs.	585.46 ± 139.37	633.97 ± 33.28
72 hrs.	360.07 ± 35.39	366.79 ± 35.12
Average over time	538.35 ± 97.48	557.42 ± 82.24

TABLE 12

EFFECT OF VITAMIN A PRESENCE IN ABSENCE
OF PROGYNON ESTRADIOL ± S. E.

Time	With Vitamin A (Vitamin A and Estradiol + Vitamin A)	Without Vitamin A (Control, Estradiol)
24 hrs.	782.96 ± 68.16	1317.06 ± 58.87
48 hrs.	750.44 ± 67.14	1135.67 ± 51.68
72 hrs.	616.85 ± 27.15	701.30 ± 63.74
Average over time	716.75 ± 118.99	1051.34 ± 199.79

TABLE 13

AVERAGE CELL NUMBERS FOR ALL TIME PERIODS FOR EACH TREATMENT WITHOUT ETHANOL-DILUTED ESTRADIOL ± S. E.

Control	678.74 ± 46.16
Vitamin A	495.49 ± 68.92
Progynon	436.09 ± 43.64
Progynon and vitamin A	581.21 ± 120.04

TABLE 14

AVERAGE CELL NUMBERS FOR ALL TIME PERIODS FOR EACH TREATMENT WITHOUT PROGYNON ESTRADIOL ± S. E.

Control	678.74 ± 46.16
Vitamin A	495.49 ± 68.92
Ethanol estradiol	1423.94 ± 70.10
Estradiol and vitamin A	938.01 ± 39.46

TABLE 15

MEAN NUMBER OF CELLS FROM ALL TREATMENTS WITHOUT ESTRADIOL FOR EACH TIME PERIOD ± S. E.

24 hrs.	670.51 ± 88.72
48 hrs.	609.71 ± 112.93
72 hrs.	363.43 ± 52.46

TABLE 16

MEAN NUMBER OF CELLS FROM ALL TREATMENTS WITHOUT PROGYNON FOR EACH TIME PERIOD ± S. E.

24 hrs.	1050.01 ± 239.73
48 hrs.	943.06 ± 220.21
72 hrs.	659.08 ± 141.70